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Anthropogenic influence on seasonal and spatial variation in bioelements and non-essential elements in honeybees and their hemolymph

Konstantin Ilijević[1], Dragana Vujanović[2], Snežana Orčić[3], Jelena Purać[3], Danijela Kojić[3], Nenad Zarić[4], Ivan Gržetić[1], Duško P. Blagojević[5], Tatjana V. Čelić[3]*

[1]University of Belgrade, Faculty of Chemistry, Studentski trg 16, Belgrade 11000, Republic of Serbia
[2]University of Belgrade, Faculty of Pharmacy, Vojvode Stepe 450, 11221 Belgrade, Republic of Serbia
[3]University of Novi Sad, Faculty of Sciences, Department of Biology and Ecology, Trg D. Obradovića 2, 21000 Novi Sad, Republic of Serbia
[4]University of Belgrade, Faculty of Biology, Studentski trg 16, 11000 Belgrade, Republic of Serbia
[5]University of Belgrade, Institute for Biological Research "Siniša Stanković", Department of Physiology, Bulevar despota Stefana 142, 11060 Belgrade, Republic of Serbia

*corresponding author: Tatjana V. Čelić, University of Novi Sad, Faculty of Sciences, Department of Biology and Ecology, Trg D. Obradovića 2, 21000 Novi Sad, Republic of Serbia
e-mail: tatjana.celic@dbe.uns.ac.rs, phone: +381214852673 fax: +38121450620

Abstract

Honeybee colony losses have been a focus of research in the last years, due to the importance of managed honeybee colonies for economy and ecology. Different unfavorable conditions from the outside environment have a strong impact on the hive health. The majority of losses occur mainly during winter and the exact reason is not completely understood. Only a small number of studies is dealing with content of bioelements, their function and influence on honeybee physiology. The aim of the present study was to determine seasonal and spatial variations in content of bioelements and non-essential elements, in hemolymph and whole body of honeybees originating from three regions with different degrees of urbanization and industrialization. Concentrations of 16 elements were compared: macroelements (Ca, K, Mg, Na), microelements (Cu, Fe, Mn, Zn) and non-essential elements (Al, Ba, Cd, Co, Cr, Ni, Pb, Sr) in samples collected from 3 different environments: Golija (rural region), Belgrade (urban region) and Zajača (industrial region). Content of bioelements and non-essential elements in honeybees was under noticeable influence of the surrounding environment, but also season and degree of honeybee activity. Hemolymph was proven to be helpful in differentiating air pollution from other sources of honeybee exposure. The results of our study demonstrated that bees can be successfully used as biomonitors since we have observed statistically significant differences among observed locations, but unless compared locations are exposed to excessively different pollution pressures, it is essential that all bees should be collected at the same season.

Keywords *Apis mellifera*, environmental pollution, hemolymph, ICP-OES, metals

1. Introduction

Honeybees (*Apis mellifera* L.) are the main pollinators of numerous plants and fruit trees, thus they have a key role in agriculture sustainability and biodiversity maintenance. However, decline of managed honeybees both in Europe and the USA, has been documented in numerous studies over the past decades, with annual loss rates ranging between 30 to 40% of the total number of managed colonies (**López-Uribe and Simone-Finstrom, 2019**). The phenomenon of the honeybee colony losses is an obvious example of how uncontrolled imbalance in the environment has direct economic consequences for human society (eg. unsustainable development) (**Gallai et al., 2009**), in addition to affecting plant diversity and ecosystem stability (**Potts et al., 2010**). Considering the importance of managed honeybee colonies for economy and ecology, colony losses have been in the focus of research in the last years. Different unfavorable conditions from the outside environment have a strong impact on the hive's health. The majority of losses occur mainly during winter and the exact reason is not completely understood. Intensive beekeeping, poor beekeeping practices and increasing environmental pollution from industry and agriculture are considered as main sources of negative pressure on honeybee health (**Goulson et al., 2015; Jacques et al., 2017**). Honeybee pests and disease, diet and nutrition, genetics, as well as cumulative, multiple exposures and/or the interactive effects of several of these factors could be the reason for colony losses (**Staveley et al., 2014; Stanimirović et al., 2019**).

Insects are exposed to mineral elements trough food and water, thus most naturally occurring mineral elements are found in their bodies. For insects, essential mineral elements, that have known metabolic function are sodium (Na), potassium (K), calcium (Ca), magnesium, (Mg), chlorine (Cl) and phosphorus (P). However, the exact amounts that insects need are not determined. Some elements are required in trace amounts for various physiological processes, but at higher concentrations, these micronutrients tend to be toxic and derange various physiological processes. Essentially, this should be viewed as part of the overall dose-response relationship for those metals shown to be essential, and the shape of this relationship can vary among organisms (**Singh et al., 2011; Tchounwou et al., 2012**). Iron (Fe) is essential as the central element in cytochrome enzymes, and zinc (Zn) and manganese (Mn) are also essential since these elements play a part in hardening the cuticle of mandibles (**Capinera et al., 2008**).

Copper (Cu) is a constituent of some enzymes and is considered essential for insects (**Gordon, 1959**). Some metals are part of vitamins or enzymes, such as cobalt (Co) and chromium (Cr), but it is unknown whether they are essential for insects, and others, such as aluminum (Al), barium (Ba), cadmium (Cd), nickel (Ni), lead (Pb) and strontium (Sr), have no known physiological function and can interfere with biological processes by interacting with macromolecules, replacing or in other ways affecting essential metals (**Buchwalter, 2008; Markert et al., 2015**).

Metals are released into the environment by natural and anthropogenic sources, and since they do not decompose, they are continuously present in the environment, changing chemical forms and entering into biological cycles (**Perugini et al., 2011**). Toxic metals in bees and in bee products have been the subject of many studies. Analyzing the literature related to metals in the environment and bees, it can be observed that most of the studies are related to different aspects of interrelations between toxic metal content in the environment, bees and bee products (**Fakhimzadeh et al., 2000; Conti and Botre, 2001; Bogdanov, 2006; Van der Steen et al., 2012; Formicki et al., 2013**). Many authors indicate the possibility of using bees and their products as bioindicators of toxic metal pollution (**Perugini et al., 2011; van der Steen et al., 2012**), and their potential in biomonitoring process **(Leita et al., 1996; Conti and Botre, 2001; Porrini et al., 2002; Celli and Maccagnani, 2003; Porrini et al., 2003; Roman, 2005; Perugini et al., 2011; Ruschioni et al., 2013; Van der Steen et al., 2015;**). Some papers deal with the influence of pollen to metals profile in honey we consume (**Lambert et al., 2012**), and some with risk assessment for humans from dietary intake of toxic metals present in the honey (**Ru et al., 2013; Fakhri et al., 2019**). In recent years, more attention was given to the influence of toxic metals to honeybee development and survival, as well as physiological and biochemical changes underlying effects of given metals (**Di et al., 2016; Hladun et al., 2016; Nikolić et al. 2016**). However, only a small number of studies have addressed the content of bioelements and their functions and influence on honeybee physiology (**Filipiak et al., 2017; Ptaszyńska et al. 2018)**.

The aim of the present study was to better understand the dynamic of bioelements and non-essential elements during an annual cycle of a beehive. In line with this objective, we determined contents of 16 elements in honeybees originating from three regions that differ in degree of urbanization and industrialization. Furthermore, this is the first study that analyzed element concentration in honeybee hemolymph. Analysis of hemolymph and atmospheric particulate matter should give better insight into ways of exposure of honeybees to environmental contaminants.

2. Materials and methods

2.1. Study sites

Adult honeybees (*Apis mellifera* L.) were collected from three regions in the Republic of Serbia with different anthropogenic impact. Detailed description of locations was previously published in **Nikolić et al. (2015).**

Apiary in Tunovo on the mountain Golija (43°17'09.7"N 20°25'44.0"E) is located in the sparsely populated rural area on the border of a biosphere reserve. Apiary in Belgrade, capitol of Serbia(44°47'37.5"N 20°27'51.3"E) is situated in large city (approximately 1.5 million inhabitants) close to two boulevards with heavy traffic. Apiary in Zajača (44°27'07.2"N 19°14'43.9"E) represents bee colonies foraging in an industrial area, since, at the time this research was conducted, there was a mining and smelting company there producing zinc, antimony and lead from primary and secondary raw materials.

2.2. Sample collection

In order to evaluate exposure to stressors resulting from colony's interaction with environment, adult forager honeybees were collected at three time points, in winter (November 2012), spring (April 2013) and summer (August 2013). Samples of forager bees were collected from five hives (approximately 20 000 bees), at each apiary.

The bees were collected from marginal frames without brood of the bee hive, in order to ensure sampling of older worker bees that already came into contact with environmental pollutants by foraging (**Medrzycki et al., 2013**). From each hive, forager bees were collected and transferred into plastic vials which were immediately frozen in dry ice and stored at -20^{o}C.

Hemolymph was collected from the same hives as honeybees. For hemolymph collection, honeybee foragers were calmed using dry ice. The bee was held with forceps, and hemolymph was collected from dorsal vascular vessel, by piercing the abdomen between tergites with sterile needle and collecting it with micropipette. Volume was approx. 1-5 μL/bee, with about 50 bees/sample. Only clear and yellow hemolymph was collected. Hemolymph was collected in 1.5 ml microtubes, kept on dry ice, and stored at -20^{o}C until further processing.

Atmospheric particulate matter (PM) sampling was performed using the bucket collection. Buckets were set near apiaries in all locations, and after two weeks deposition particles were collected, filtered and further processed for elemental analysis.

2.3. Sample preparation

Samples of honeybees were dried at 55 ± 5°C until constant mass was reached, after which they were homogenized. Particulate matter (PM) was dried at room temperature for 24h, sieved through 160μm sieve and then dried at 100 ± 5°C until constant mass. Hemolymph samples were in liquid state and were not dried.

Digestion was performed in closed microwave digestion system (ETHOS 1, Advanced Microwave Digestion System, Milestone, Italy) in accordance with the US EPA SW-846 Method 3052. Samples, mass of approximately 0.5000 g or less, were transferred to closed Teflon vessels and digested with concentrated HNO_3 and concentrated H_2O_2 (volume ratio in ml for whole body of bees, hemolymph and PM were 6:2, 5:1 and 7:1 respectively).

Samples were gradually heated from room temperature to 200 °C during 15 minutes, and then kept at 200 °C for 10 minutes for hemolymph and 15 minutes for the whole body of honeybees and PM. If necessary, digestate was filtered through Sartorius 389 filter paper (white ribbon) to remove refractory silicate particles. All samples were diluted to the known volume (10, 25 or 100 ml) with double deionized water.

2.4. Instrumental analysis

Concentrations of: Al, Ba, Cd, Co, Cr, Cu, Ca, Fe, K, Mg, Mn, Na, Ni, Pb, Sr and Zn were measured by inductively coupled plasma optical emission spectroscopy, ICP-OES (iCAP 6500Duo, Thermo Scientific). Very low concentrations of: Co, Cr, Cd and Pb, which occurred in hemolymph were confirmed by another, more sensitive analytical technique, inductively coupled plasma mass spectroscopy, ICP-MS (iCAP-Q-ICP-MS, Termo Scientific). Standard solutions were prepared from multi-element plasma standard solution 4, Specpure®, Alfa Aesar.

Quality control was based on the analysis of blanks (prepared following the whole sample preparation procedure) and analysis of the standard solutions (with low, medium and high concentrations) at the beginning, after every 10 samples, and at the end of the analysis. Samples were prepared and analyzed in triplicate.

2.5. Statistical analysis

Statistical analysis was performed using STATISTICA v13 software. The effect of two factors, different sampling location and season on concentration of elements in honeybee foragers was analyzed using factorial ANOVA. Significant differences were estimated with $p < 0.05$, $p < 0.01$ and $p < 0.001$ confidence intervals. In order to have an insight about influence of season and location on concentration of elements, one-way ANOVA followed by post-hoc Tukey test was performed for each factor. Significant differences were estimated with $p < 0.05$ confidence intervals.

3. Results and discussion

We have compared concentrations of elements determined in samples collected from 3 different environments: rural region (Golija), urban region (Belgrade) and industrial region (Zajača). In order to more easily interpret the results, elements were divided into three groups: macroelements (Ca, K, Mg, Na), microelements (Cu, Fe, Mn, Zn) and non-essential elements (Al, Ba, Cd, Co, Cr, Ni, Pb, Sr).

3.1. Annual concentrations of elements in the whole body of honeybees

Annual values were calculated from element concentrations measured over the entire study period. Mean, minimum and maximum annual values and standard deviations of element concentrations in the whole body of honeybees are shown in Table 1. For most bioelements (macro and microelements), average annual concentrations were not significantly different between locations, except for Fe which was higher in Belgrade in comparison with other two locations. Statistical significance was not observed probably because of the seasonal variations in content of bioelements, causing large standard deviations.

Table 1. Mean, minimum, maximum values (mg kg^{-1} d.m.) and standard deviation for annual values of elements concentration in the whole body of forager bees for three regions: Golija (rural region), Belgrade (urban region) and Zajača (industrial region).

		Golija				**Belgrade**				**Zajača**			
		Mean	**Min**	**Max**	**St.dev.**	**Mean**	**Min**	**Max**	**St.dev.**	**Mean**	**Min**	**Max**	**St.dev.**
Macroel.	**Ca**	**677.54**^{a}	406.02	937.32	222.20	**658.46**^{a}	456.76	780.92	152.85	**822.29**^{a}	525.77	1410.68	303.87
	K	**2416.79**^{a}	2049.54	2570.02	195.01	**2914.32**^{a}	2773.47	3223.06	169.67	**3058.77**^{a}	2139.38	4899.69	991.78
	Mg	**705.94**^{a}	552.42	918.39	165.72	**711.93**^{a}	573.12	1035.74	148.89	**670.39**^{a}	520.46	780.30	100.40
	Na	**200.66**^{a}	75.16	367.87	115.34	**241.68**^{a}	119.29	447.42	141.55	**257.27**^{a}	124.60	465.69	117.32
Microel.	**Cu**	**16.84**^{a}	9.36	21.23	4.55	**23.26**^{a}	15.53	34.57	6.37	**16.44**^{a}	10.82	23.94	5.61
	Fe	**82.87**^{a}	55.51	141.09	29.14	**128.06**^{b}	75.33	184.25	40.10	**71.35**^{a}	36.76	94.62	25.16
	Mn	**87.26**^{a}	50.06	139.01	33.75	**43.38**^{a}	30.37	54.47	10.15	**80.00**^{a}	33.84	223.00	68.45
	Zn	**80.81**^{a}	66.36	113.90	15.65	**86.16**^{a}	70.66	103.59	10.64	**93.53**^{a}	56.34	202.49	48.49
Non-essential elements	**Al**	**23.34**^{a}	4.15	69.84	22.24	**32.95**^{a}	11.32	73.91	25.22	**18.52**^{a}	4.21	42.60	17.41
	Ba	**3.86**^{a}	1.42	6.32	1.92	**2.30**^{a}	0.94	4.53	1.28	**3.63**^{a}	1.53	6.90	2.02
	Cd	**0.59**^{a,b}	0.08	1.47	0.56	**0.17**^{a}	0.15	0.20	0.02	**1.57**^{b}	0.29	4.95	1.69
	Co	**0.29**^{a}	0.10	0.44	0.14	**0.14**^{b}	0.09	0.20	0.04	**0.13**^{b}	0.06	0.26	0.08
	Cr	**0.20**^{a}	0.15	0.23	0.03	**0.24**^{a}	0.20	0.28	0.03	**0.23**^{a}	0.17	0.31	0.08
	Ni	**0.57**^{a,b}	0.26	0.85	0.22	**0.69**^{a}	0.42	1.08	0.22	**0.34**^{b}	0.15	0.53	0.11
	Pb	**0.42**^{a}	0.26	0.57	0.16	**0.43**^{a}	0.20	0.71	0.15	**20.38**^{b}	11.63	36.90	7.12
	Sr	**4.51**^{a}	0.93	7.80	2.95	**1.12**^{b}	0.67	1.77	0.50	**1.30**^{b}	0.73	2.00	0.49

Different letters indicate statistically significant differences between metal concentrations in samples from different locations.

The average annual values for most non-essential elements, except Al, Ba and Cr, showed differences between locations. The obtained values for non-essential elements are in accordance with other studies of metal contents in honeybees, a detailed review of which was given in the work of **van der Steen et al. (2012)**, and also available in other literature data (e.g. **Perugini et al., 2011; Matin et al. 2016**; **Zarić et al., 2016, 2017**). Bee samples from Golija had significantly higher annual concentrations of Co and Sr. Concentration of Pb in the whole body of bees was extremely elevated in Zajača, compared to Belgrade and Golija, and compared with literature data (**Roman, 2005; Perugini et al., 2011; van der Steen et al. 2012; van der Steen et al. 2015; Zarić et al., 2016, 2018**). Concentration of Cd was considerably higher, while concentration of Ni was significantly lower in bees from Zajača than in bees from Belgrade.

3.2. Seasonal and spatial variations of element concentration in the whole body of honeybees

Results of factorial ANOVA used for evaluating the effect of different location (Golija, Belgrade, Zajača) and season (spring, summer, winter) on concentration of elements in the whole body of bees are presented in Table S1. Most of the elements were influenced by both location and season, and combined influence of both factors. The exception were Mg and Zn whose concentrations were not influenced by location, and Mn whose concentration was not affected by season. Factorial ANOVA could not be performed for Cr, since concentration of this element was under detection limit in several samples.

3.2.1 Seasonal variations

Element concentrations in different seasons (winter, spring and summer) in whole body of bees from three locations is presented in Table 2. Descriptive statistics (mean, minimum, maximum and standard deviation) for each location is available as Supplement tables S2, S3 and S4.

Concentrations of bioelements: Ca, K, Na and Fe were the highest during summer months in bees from all investigated locations, Mg had the highest concentrations during summer months in bees from Golija and Belgrade, but in Zajača Mg concentration was the highest during winter. In Zajača, K, Na and Fe had tendency to remain high in winter months as well. Concentrations of Cu, Mn and Zn had different seasonal patterns in bees from different locations. Most bioelements were higher in period of intense bee activity i.e. spring or summer. Since winter bees have longer lifespan than summer bees, and have developed certain physiological adaptations associated with longevity (**Amdam and Omholt, 2002; Orčić et al., 2017**), changes in concentrations of bioelements are probably correlated with physiological changes and lower activity of winter bees. **Ptaszyńska et al. (2018)** published a study about bioelement concentrations in winter and summer bees, and concluded that most of bioelements (Ca, Cu, K, Mg, Zn) are higher during summer, while during winter the highest concentrations are observed for Fe and Mn. In this study, different trends for some metals were found. Macroelements Ca, K and Mg have higher concentrations during summer in both studies, but microelements show discrepancies. Copper did not show regularity at three locations, while zinc and manganese had higher concentrations during winter only in Zajača. Iron had higher concentrations during summer in bees from Golija and Belgrade. Differences in bioelements could be the result of different plants that bees visit to collect pollen and nectar, different water sources, and also potential supplementation with sugar syrup during winter (**Filipiak et al., 2017**). Nevertheless, there is also a possibility that bioelements may appear in elevated concentrations as a consequence of pollution since they are often released into the environment along with other nonessential elements. It would be necessary to analyze more samples before it can be safely claimed that differences in contents of bioelements have certain seasonal regularities.

The highest concentrations of non-essential elements, in general, occurred during spring and summer. This was expected considering that honeybees rarely leave the beehive during winter, thus contact with environmental pollutants is minimized. Presumed source of metals during winter is food collected during active season (**Döke et al., 2015**). However, some toxic metals had specific patterns in seasonal trends at each analyzed site. Toxic metal concentrations in bees collected from Golija were higher during spring and summer, while in winter they were similar to the values determined at other two analyzed sites. Such trend was discovered for: Al, Ba, Cd, Co, Cr and Sr. Trends in Belgrade were similar, except for Cd which had a constant concentration throughout the year. However, bees from Zajača had the highest concentrations of Cd and Co during winter. Concentration of Pb remains high during the whole year, compared to other two locations and presents a significant risk to honeybee colonies from this locality.

Table 2. Mean concentration of elements (mg kg^{-1} d.m.) in the whole body of forager bees given by season of the year for three regions: Golija (rural region), Belgrade (urban region) and Zajača (industrial region).

		Golija			**Belgrade**			**Zajača**		
		Winter	**Spring**	**Summer**	**Winter**	**Spring**	**Summer**	**Winter**	**Spring**	**Summer**
Macroel.	**Ca**	409.74^{a}	716.30^{b}	906.59^{c}	457.11^{a}	744.80^{b}	773.46^{b}	715.36^{a}	555.35^{a}	1196.15^{b}
	K	2536.92^{a}	2296.65^{a}	>OR	3037.37^{a}	2791.28^{a}	>OR	3318.61^{a}	2185.28^{b}	4899.69^{c}
	Mg	565.30^{a}	635.52^{a}	917.00^{b}	574.07^{a}	716.14^{a,b}	845.59^{b}	767.67^{a}	543.50^{b}	700.01^{c}
	Na	190.28^{a}	75.37^{b}	336.33^{c}	159.65^{a}	136.28^{a}	429.12^{b}	281.78^{a,b}	131.23^{a}	358.81^{b}
Microel.	**Cu**	19.93^{a}	10.91^{b}	19.67^{a}	22.50^{a}	16.96^{a}	30.31^{b}	23.79^{a}	11.66^{b}	13.87^{c}
	Fe	58.51^{a}	72.55^{a}	117.56^{b}	77.79^{a}	140.80^{b}	165.60^{c}	81.75^{a}	38.57^{b}	93.72^{c}
	Mn	56.05^{a}	114.03^{a}	91.70^{a}	30.44^{a}	50.13^{b}	49.58^{b}	164.20^{a}	35.18^{b}	40.62^{b}
	Zn	68.63^{a}	92.67^{a}	81.12^{a}	94.21^{a}	87.61^{a}	76.66^{a}	149.86^{a}	61.65^{b}	69.09^{b}
Non-essential elements	**Al**	4.17^{a}	19.26^{a,b}	46.58^{b}	11.34^{a}	64.62^{b}	22.89^{a}	4.26^{a}	9.82^{b}	41.49^{c}
	Ba	1.51^{a}	4.43^{b}	5.65^{b}	0.98^{a}	2.17^{a}	3.76^{b}	3.00^{a}	1.72^{b}	6.17^{c}
	Cd	0.08^{a}	1.29^{b}	0.42^{a}	0.19^{a}	0.17^{a}	0.16^{a}	3.69^{a}	0.63^{b}	0.39^{b}
	Co	0.11^{a}	0.40^{b}	0.37^{b}	0.09^{a}	0.12^{a,b}	0.19^{b}	0.2^{a}	<DL	0.07^{b}
	Cr	<DL	0.17^{a}	0.22^{a}	<DL	0.24^{a}	0.24^{a}	<DL	<DL	0.23
	Ni	0.68^{a}	0.29^{b}	0.76^{a}	0.44^{a}	0.79^{a,b}	0.85^{b}	0.23^{a}	0.37^{a}	0.41^{a}
	Pb	<DL	<DL	0.42	0.27^{a}	0.55^{b}	0.45^{a,b}	17.50^{a}	15.96^{a}	27.68^{a}
	Sr	0.93^{a}	7.70^{b}	4.90^{c}	0.67^{a}	0.92^{b}	1.76^{c}	1.37^{a,b}	0.80^{a}	1.72^{b}

Different letters indicate statistically significant differences between metal concentrations in samples taken in spring, summer and winter at the same location.

OR- operating range; DL- detection limit.

This suggests that, during summer, honeybees from Zajača collected and stored food with high concentrations of toxic elements. Hence, winter bees feeding with this food accumulated toxic elements in their bodies. Since bees rarely defecate during winter, this can contribute to metal accumulation, which could lead to physiological alterations and disturbance in homeostasis (**Nikolić et al. 2015, 2016; Di et al., 2016; Hladun et al., 2016**).

3.2.2. Spatial variations

Mean concentrations comparison of elements in the whole body of bees from different locations are shown in Figure 1. Analysis of element concentrations in the whole body of bees implies that each of the three analyzed environments imposes specific pollution pressure to the honeybee population that inhabits it. Determined concentrations are a consequence of combined influences of bees' interactions with their environment (deposition of particles on the surface of bees bodies, intake by inhalation and nourishment) and bees ability to accumulate metals or block their absorption.

Differences in content of bioelements are probably a consequence of different floral plant species presented at studied locations, as well as elemental content of water that bees collect around their hives. Winter bees from Zajača had the highest concentration of bioelements, while in spring bees the highest concentrations were measured in Belgrade. In summer bees, differences among locations were not so noticeable. Bees collected from the urban environment of Belgrade had higher concentrations of Cu and Fe in spring and summer in comparison with other locations. Sources of these elements are probably related to traffic (they can be released by wearing of tires and brakes) or to fossil fuel combustion (**Duong et al., 2011; Al Khashman, 2013**).

Differences in toxic elements concentrations among locations changed in relation to season. Honeybees from Golija were exposed to the highest concentrations of Ba, Cd, Co, Ni and Sr, bees from Belgrade had elevated concentrations of Al, Cr and Ni, while bees from Zajača had elevated concentrations of Ba, Cd, Pb and Sr, during at least one season.

Golija was considered as "clean" location, with no sources of pollution in the area covered by bees during foraging. However, this study established that bees from rural environment can also be under substantial pollution pressure from toxic elements. Concentrations of toxic elements in forager bees were the same as in industrial area, Zajača, and for certain elements (Ba, Cd, Co and

Sr) even significantly higher. It is possible that cadmium and other non-essential elements are naturally occurring in soil from Golija. They can also come via long range transport of aerosol from distant pollution sources. For example, cadmium from soil is transported to plants and can contaminate nectar and pollen, and only a small amount of this metal gets into the bee's products from air, and only if it is in close proximity to sources of pollution (**Bogdanov, 2006**).

Bees from Belgrade had high concentrations of Al, particularly in spring, and Ni in spring and summer. Al is one of the most abundant elements in Earth crust, which indicates that it is probably of natural origin (**Adokoh et al., 2011**), while high concentration of Ni is probably the consequence of intense traffic in urban areas (**Duong et al., 2011**). It was interesting to notice that bees from Belgrade and Golija had similar levels of Pb, which suggests that exclusion of leaded gasoline in recent years significantly decreased the amount of bioavailable lead in urban areas.

Considering that there was a lead smelter company in Zajača, Pb was regularly monitored by the Environmental Protection Agency of the Republic of Serbia (**Popović et al., 2014**). They have found extremely elevated concentration in the air surrounding the bee collection site. It is known that plants do not transport lead, so bees are thought to come in contact with this metal through air (**Bogdanov, 2006**). Hence, very elevated Pb concentration in honeybees at Zajača was a consequence of the lead smelter company emitting high Pb pollution in the surrounding air.

3.3. Hemolymph

Hemolymph samples were collected in winter and summer, simultaneously with honeybee foragers, and mean concentrations of elements are presented in Table 3. Hemolymph is a body fluid of invertebrates that transports nutrients and other substances, and maintain internal environment for cells through its regulation of ionic and chemical composition. It is a dynamic tissue that changes with the changing physiological state of the insect (**Klowden, 2013**). Unlike the whole body of honeybees, which might contain some small amount of metals whose source is PM attached to the hairy surface of the bee, concentrations of metals in hemolymph are regulated only by metabolic processes. Actual concentration of elements in hemolymph depends on honeybee exposure to these elements present in their environment, but also on the ability of bees to block their absorption, ability to successfully excrete them, and on tendencies to accumulate certain element in their bodies.

Table 3. Concentration of elements (mg kg^{-1}) in hemolymph of forager bees for three regions: Golija (rural region), Belgrade (urban region) and Zajača (industrial region), collected in winter and summer.

		Winter			Summer		
		Golija	**Beograd**	**Zajača**	**Golija**	**Beograd**	**Zajača**
Macroel.	**Ca**	87.35	139.97	114.5	236.80	311.62	192.51
	K	581.16	920.22	869.56	940.58	3568.80	1149.90
	Mg	87.01	135.41	82.08	208.85	372.81	172.91
	Na	198.02	357.06	160.61	442.02	1105.06	551.28
Microel.	**Cu**	1.80	3.65	1.47	2.66	8.10	2.43
	Fe	3.20	1.36	1.55	5.52	13.49	10.36
	Mn	0.78	0.89	3.62	0.63	1.19	0.74
	Zn	9.33	12.98	6.82	12.33	15.14	6.62
Non-essential elements	**Al**	0.97	0.84	1.04	0.52	0.63	21.77
	Ba	0.40	0.18	0.06	2.46	6.94	3.83
	Cd	0.05	<DL	0.12	<DL	<DL	<DL
	Co	<DL	<DL	<DL	0.05	<DL	0.17
	Cr	<DL	<DL	<DL	0.03	0.05	0.03
	Ni	0.04	<DL	<DL	0.31	0.61	0.33
	Pb	<DL	<DL	0.91	0.24	0.71	1.41
	Sr	<DL	<DL	0.07	0.16	0.27	0.20

DL- detection limit.

Literature data about element concentration in hemolymph of honeybees are lacking, probably because of the complicated sampling and requirements of large volumes of hemolymph for this analysis. To the best of our knowledge this is the first study to determine elemental composition of hemolymph. Concentrations of bioelements as well as non-essential elements were lower in hemolymph than in the whole body of honeybees from same locations and seasons (Table 2 and 3). Among bioelements, only exception was Na whose concentrations were higher in hemolymph than in the whole body of bees. Concentrations of Ba and Ni were in the same order of magnitude in both matrices. On the other side, concentrations of Al, Cd, Cr, Cu, and Sr were for at least one order of magnitude lower in the hemolymph.

Sodium is one of the major inorganic components of the body fluids, but in insects potassium could have more important role than sodium. Furthermore, plant-feeding insects tend to have higher levels of potassium than insect feeding on other diets, that have higher levels of sodium (**Klowden, 2013**). Although honeybees are feeding on plants, it is possible that sodium is major electrolyte in their hemolymph, and thus its concentration is higher than in the whole body of bees.

Seasonal differences of element concentration in hemolymph were similar as in the whole body of bees. In general, elemental concentrations were lower during winter in samples from all locations, and most of the non-essential elements were below detection limit in samples collected during winter, with some exceptions: in samples from Golija, concentration of Al and Cd were higher during winter, as well as concentration of Mn and Cd in hemolymph samples from Zajača. Spatial differences in non-essential elements concentration were similar in whole body of bees and hemolymph during winter, except for Ba, while during summer, spatial differences of non-essential element concentrations in the whole body of bees was not followed by the same trends in hemolymph, except for Ni and Pb, which suggests that airborne particulate matter is dominant way of exposure of honey bees to pollution.

3.4. Particulate matter (PM)

Samples of PM collected during summer showed differences between locations (Table 4). Samples of PM collected at Golija had the highest concentrations of K, Mg, Fe, Mn, Co, Cr and Sr. Samples from Belgrade had the highest concentrations of Cu, Ba and Ni. Samples of PM collected in Zajača had higher concentrations of Na and Zn and many times higher concentrations of Al, Pb and Cd compared to samples from other two locations.

Comparison of some elements showed similar spatial differences in the whole body of bees and PM, while for others those similarities were not observed. Namely, spatial differences in content of Cu, Mn, Co, Cr, Ni, Pb and Sr were similar in both matrices, so it can be concluded that concentrations of these elements in bees could be the result of deposition of dust particles on their bodies, which are covered with hair, and therefore prone to collect PM. For all macroelements, as well as for Fe, Zn, Al, Ba and Cd, there were no similar spatial differences in the whole body of bees and PM, which could mean that main source of these elements is not deposition of PM from atmosphere, but rather pollen, nectar and water. Furthermore, comparison of spatial differences in concentration of bioelements in PM and hemolymph did not show similarities, which is probably the result of regulatory mechanisms of hemolymph content. Concentration of some non-essential elements, Al, Ba, Ni and Pb showed similar spatial differences in both matrices, while Cd, Co, Cr and Sr did not showed similarities, which gives more insight into potential sources of contamination and mechanisms of elimination of non-essential elements from hemolymph. Nevertheless, these assumptions should be confirmed with analysis of all potential pollution sources around beehives.

Table 4. Concentration of elements (mg kg^{-1} d.m.) in particulate matter collected in summer 2013 from three regions: Golija (rural region), Belgrade (urban region) and Zajača (industrial region).

		Particulate matter		
		Golija	**Belgrade**	**Zajača**
Macroel.	**Ca**	>OR	16498.63	9199.26
	K	6183.62	3065.34	2655.37
	Mg	6574.05	6150.83	2356.98
	Na	702.63	273.98	807.52
Microel.	**Cu**	57.89	139.37	115.91
	Fe	23765.19	11949.67	19375.87
	Mn	483.60	289.78	449.08
	Zn	406.61	744.42	1107.74
Non-essential elements	**Al**	30583.33	17872.36	87174.89
	Ba	288.99	427.34	159.44
	Cd	3.23	1.72	73.19
	Co	13.16	11.7	8.07
	Cr	65.59	61.16	57.83
	Ni	51.61	93.03	64.66
	Pb	182.99	117.99	12471.21
	Sr	150.23	38.25	24.71

OR- operating range.

4. Conclusion

Content of bioelements and non-essential elements in honeybees is under noticeable influence of their environment, but also season and activities of honeybees. The results of our study demonstrated that bees can be successfully used as biomonitors since we have observed statistically significant differences among studied locations. However, unless compared locations are exposed to excessively different pollution pressures, it is essential that all bees are collected during the same season. This conclusion is based on the fact that significant seasonal differences are observed in all locations, for bioelements as well as for non-essential elements.

Although some pristine locations may seem unpolluted, bees from these areas could still be under significant exposure to toxic elements which are present in high background concentrations derived from natural weathering of local geological materials or long-range pollution transfers.

Bees collected in summer and spring had higher concentrations of bioelements as well as non-essential elements content in the whole body, which was expected because bees are more active in these periods of year than in winter. Increased honeybee activity probably leads to elevated concentrations of investigated elements. Contrary to this trend, at industrial sampling location it was observed that several bio (Mg, Cu, Mn, Zn) and non-essential (Cd, Co) elements had the highest concentrations during winter, which could be a consequence of different plants available at this location and higher elemental content in food that bees consume during winter. Our results suggest that, in highly polluted areas, bees can be significantly affected by airborne pollution even during their periods of lower activity and overwintering, which has important implications for the survival of the bees colonies, but also for the bees potential to serve as biomonitors.

Analysis of hemolymph provided better understanding of dynamic in changes of elemental content during an annual cycle of a beehive, which were exposed to toxic elements. It was observed that hemolymph had significantly higher concentrations of both bioelements and non-essential elements during summer months compared to winter period. The same was observed at all investigated locations (rural, urban and industrial). Therefore, it can be concluded that elevated concentrations of bioelements, but also non-essential (toxic) elements which were observed in analyzed bees during spring and summer are mainly a consequence of their intake via consumed food and water.

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Figure captions

Figure 1. Mean concentration (shown in *log* scale) comparison of all analyzed elements for three regions: Golija (rural region), Belgrade (urban region) and Zajača (industrial region).

Different small case letters represent statistically significant differences in element concentrations between locations in the same season of the year.

OR- operating range; DL- detection limit.