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EFFECTS OF METALS ON BLOOD OXIDATIVE STRESS BIOMARKERS AND ACETYLCHOLINESTERASE ACTIVITY IN DICE SNAKES (*NATRIX TESSELLATA*) FROM SERBIA

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Abstract - The effects of waterborne metals in water on the activities of blood copper-zinc superoxide dismutase (CuZn-SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR), glutathione-S-transferase (GST), and acetylcholinesterase (AChE), and on the concentrations of total glutathione (GSH) and lipid peroxides (TBARS) in the blood of dice snakes (*Natrix tessellata*) caught in Obedska Bara, Sebia (control area), with snakes caught in Pančevački Rit, a contaminated area in Serbia were examined. The activities of CAT, GSH-Px, GR and AChE, and the concentration of TBARS were significantly decreased, while GST activity and GSH concentration were significantly increased in snakes from the contaminated area compared to specimens from the control area. Significantly increased concentrations of Al, As, B, Ba, Ca, Cu, Fe, K, Li, Mn, Na, Ni and Zn in the water at the contaminated area as compared to control area were detected. The metals Ag, Bi, Cd, Co, Hg, In and Tl were not observed in any of the localities. Cr, Mo and Pb were not detected at the control area but were observed at the contaminated area. The concentrations of Sr were similar at both sites. The concentration of Mg was 2-fold higher at the control site than at the contaminated area. The obtained results show that most of the investigated blood biomarkers correlate with concentrations of metals present in the environment. These findings suggest that dice snakes are sensitive bioindicator species for monitoring the effects of increased metal concentrations in the environment.

Key words: Oxidative stress biomarkers; acetylcholinesterase; *Natrix tessellata*; metals; pollution

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INTRODUCTION

Many environmental contaminants or their metabolites, including metals, have toxic effects associated with oxidative stress, and a set of biomarkers have been developed for assessing environmental quality (Regoli and Principato, 1995; Van der Oost et al., 2003). Oxidative stress parameters have been proposed as useful biomarkers of environmental contaminants in a variety of marine and freshwater organisms (Borković et al., 2005). These biomarkers include enzymatic components, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR) and phase II biotrans-

formation enzyme glutathione-S-transferase (GST), as well as non-enzymatic components, such as total glutathione (GSH), lipid peroxides (TBARS), and many others. The responses of individual biomarkers to oxidative stress depend on the types of organism and pollutants, and this variability is generally high in field conditions. In addition to oxidative stress, pollution-related neurotoxicity in aquatic organisms can occur. Acetylcholinesterase (AChE) is one of the most common biomarkers of neurotoxicity in aquatic organisms (Durieux et al., 2011). This enzyme is responsible for the hydrolytic decomposition of the neurotransmitter acetylcholine in postsynaptic membranes. It is inhibited by various toxic compounds (Xuereb et al., 2009). Responses to the presence of stressors in the environment, such as the antioxidant response and neurotoxic effects, are observed before other disorders such as disease, mortality or population changes. Thus, they provide "early warning signals" to the presence and impact of pollutants (Ben Ameur et al., 2012).

While many biomonitoring studies have employed mollusks, fish or mammals, little is known about the responses of reptile species, and especially snakes, to the exposure to and the effects of different contaminants, especially metals. Snakes are considered to be an ideal species for monitoring contaminants in the wild (Rezaie-Atagholipour et al., 2012). All snakes are carnivores and are known to accumulate environmental contaminants (Wylie et al., 2009). Aquatic snakes are comparatively long-lived organisms. They occupy middle or higher positions in the food chain and inhabit a limited range of habitats in comparison to some mammals and most birds. Snakes have proven to be useful for making comparisons between multiple sites, e.g., control sites and contaminated sites (Burger et al., 2007). Although long-living reptiles are very suitable for biomonitoring studies, snakes are the least studied group of vertebrates in environmental risk assessment (Hopkins, 2000). Their omission in ecotoxicological studies likely stems from their life history; they produce small numbers of offspring at short intervals and are difficult to maintain in captivity (Campbell and Campbell, 2001).

The dice snake (*Natrix tessellata* Laurenti, 1768) is a non-venomous colubrid species with a wide distribution range that extends from Italy to northern Egypt and northwestern China. The most northerly European populations of these snakes were recorded in Germany and the Czech Republic (Vlček et al., 2010). The dice snake is a semi-aquatic species and almost exclusively piscivorous (Filippi et al., 1996). Egg laying usually takes place in July, and one clutch consists of 4-29 eggs (Luiselli and Rugiero, 2005). Young snakes hatch in early September. Dice snakes hibernate from October to April in dry holes near the water. Prehibernation is characterized by the accumulation of fat which provides the main source of energy as preparation for the upcoming cold period.

The aim of this study was to examine differences between oxidative stress biomarkers, (activities of CuZn SOD, CAT, GSH-Px, GR and GST and concentration of GSH and TBARS), and a neurotoxicity biomarker (AChE activity), in the blood of dice snakes, resulting from extended exposure to dissolved metals in the aquatic environment. This study was performed on snakes from the control site at Obedska Bara, and a contaminated site at Pančevački Rit in northern Serbia. The localities are characterized by different concentrations of metals in the water and therefore expected to elicit different responses in snakes.

MATERIALS AND METHODS

Animal Procedures

All animal procedures complied with the European Directive (2010/63/EU) on the protection of animals used for experimental and other scientific purposes, and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade.

Site description and sample collection

Snakes were caught in autumn (late October) at two sites: Obedska Bara (control area) and Pančevački Rit (contaminated area) (Fig. 1). The nature reserve

Fig. 1. Geographical positions of the studied localities. Obedska Bara: Latitude: 44°44.8'08.37" N; Longitude: 19°59'14.38" E; Pančevački Rit: Latitude: 44°50'01.68" N; Longitude: 20°29'48.43" E in Serbia.

Obedska Bara (44°44.8'08.37" N; 19°59'14.38" E) is a region protected under the Ramsar Convention as a wetlands of international importance and includes the rest of the abandoned bed of the Sava River. Obedska Bara is connected to the Sava River only when the water levels are elevated. It is one of the oldest protected areas in the world (since 1874), covering an area of about 20,000 ha. Pančevački Rit (44°50'01.68" N; 20°29'48.43" E) is located in the southwestern part of Banat near Belgrade, between the rivers Danube and Tamiš (Fig. 1). In the past, it was wetland area that was often flooded by the Danube and Tamiš. Today it is a suburban region exposed to increased anthropogenic pressure, receiving extensive industrial and urban waste discharges. Consequently, it is exposed to a variety of contaminants, communal and industrial waste from a factory for glue production, cattle fodder, dairy farm, sugar refinery, slaughter house, glass and chemical industries and the Pančevo oil refinery.

Ten and 7 snake specimens were caught at Obedska Bara and Pančevački Rit, respectively. The snakes from Obedska Bara were 67.7 ± 4.26 cm SVL (snout-vent length) and weighed 131.7 ± 30.77 g. The snakes from Pančevački Rit were 60.2 ± 0.90 cm SVL and weighed 77.9 ± 7.91 g. All captured snakes were adult females and in good body condition. Animal capture was approved by the Serbian Ministry for Energy, Development and Environmental Protection (Permissions Nos: 353-01-640/2012-03 and 353-01-77/2013-08) and handled in accordance with the guidelines of the Animal Welfare Act of the Republic of Serbia.

Determination of oxidative stress parameters

The animals were euthanized by decapitation and fresh blood was immediately collected, using heparin (5 000 U/mL) as an anticoagulant, and centrifuged (5 000 rpm) to separate the plasma and blood cells. Isolated red blood cells (RBCs) were washed three times with 3 vol. of 0.65% NaCl.

The hemoglobin (Hb) concentration was determined by the cyanmethemoglobin method (Drabkin

and Austin, 1935). Measurement of CuZn SOD activity was conducted in the hemolysates of washed RBCs from which Hb was previously removed by the method of Tsuchihashi (1923). CuZn SOD activity was measured by the epinephrine method (Misra and Fridovich, 1972), based on the capacity of SOD to inhibit autoxidation of adrenaline to adrenochrome. One unit of SOD activity was defined as the amount of hemoglobin (Hb) that caused 50% inhibition of the autoxidation of adrenaline.

CAT activity was assayed as suggested by Claiborne (1984), and expressed as mmol H_2O_2/min/g Hb. The method is based on H_2O_2 degradation by the action of CAT contained in the examined samples. In this procedure, 30 mM H_2O_2 served as the substrate.

Hemolysates containing about 50 g Hb/L were prepared according to McCord and Fridovich (1969) and used for the determination GSH-Px activities according to the method of Maral et al. (1977), which is based on the measurement of nicotine amide dinucleotide phosphate (NADPH) consumption, and expressed as nmol NADPH/min/g Hb.

The activity of GR was determined by measuring NADPH oxidation at 340 nm in the presence of oxidized glutathione (Glatzle et al., 1974). The method is based on the ability of GR to catalyze the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) using NADPH as substrate in phosphate buffer (pH 7.4).

For the determination of GST activity in the plasma, 1-chloro-2,4-dinitrobenzene (CDNB) was used as a substrate (Habig et al., 1974). The method is based on the reaction of CDNB, with the -SH groups of glutathione catalyzed by GST contained in the samples. One unit of GST activity was defined as nmol GSH/min/mL of plasma.

The concentration of total GSH in the plasma was measured by a standard method according to Griffith (1980). The concentration of blood lipid peroxides (LP) was estimated by the thiobarbituric acid reactive substances (TBARS) assay as described previously (Ohkawa et al., 1979). Malonic dialdehyde is a product of lipid peroxidation, and it was identified in the reaction with thiobarbituric acid, which produces a red-colored mixture with absorbance at 535 nm.

The activities of antioxidant defense enzymes were measured using a Shimadzu UV-160 spectrophotometer and a temperature-controlled cuvette holder. All chemicals were obtained from Sigma-Aldrich (St Louis, MO,USA).

Determination of acetylcholinesterase (AChE) activity

The activity of AChE was determined in the plasma using the Ellman method (Ellman et al., 1961). The assay involves a reaction of 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) with thiocholine liberated from its esters by enzymatic hydrolysis. The yellow 5-thio-2-nitrobenzoate (TNB) that is formed was detected spectrophotometrically at 412 nm.

Trace metal concentrations in water

The following 25 trace metals were determined in the water at both localities: Ag, Al, As, B, Ba, Bi, Ca, Cd, Co, Cr, Cu, Fe, Hg, In, K, Li, Mg, Mn, Mo, Na, Ni, Pb, Sr, Tl and Zn. Water samples were collected, pre-filtered in the field and stabilized with nitric acid at pH below 2. Analysis of the total element content in water samples was performed by the method for preparation given in U.S. EPA (2007). Their concentration was determined by ICP-OES (Spectro Genesis EOP II, Spectro Analytical Instruments DmbH, Kleve, Germany). For the validation of analytical procedures, we used a standard water reference (NIST-1643, RS stimulated fresh water trace elements). Analysis of all samples was performed in five sample replicates (n = 5) and the concentrations were expressed in μg/L (ppb).

Statistical analysis

The data are expressed as the means ± standard error (SE). The non-parametric Mann-Whitney U-test

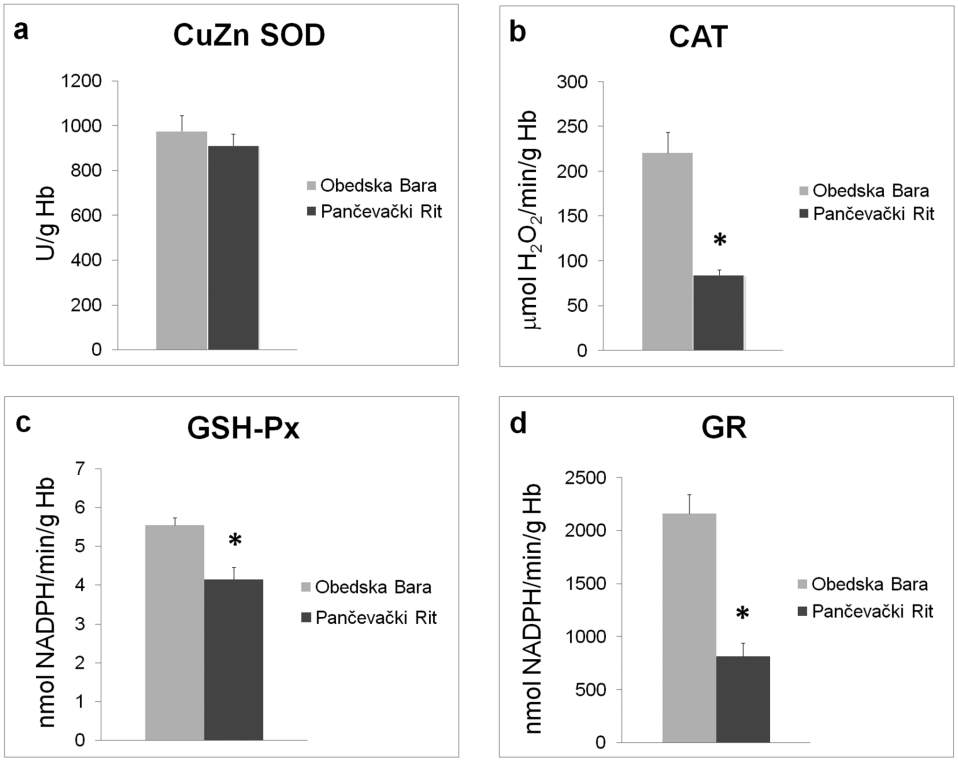

Fig. 2. Comparison of specific antioxidant enzyme activities in snakes from different localities. **A** – copper-zinc containing superoxide dismutase (CuZnSOD); **b** – catalase (CAT); **c** – glutathione peroxidase (GSH-Px); **d** – glutathione reductase (GR) in RBCs of the dice snake (*Natrix tessellata*) from Obedska Bara and Pančevački Rit. Specific enzyme activities are expressed as U g^{-1} hemoglobin (Hb). The samples were compared using the Mann-Whitney *U*-test; (*) significant difference was at $p<0.05$. The number of different specimens used to calculate the means: N=10 for Obedska Bara and N=7 for Pančevački Rit.

was performed to assess significant differences between samples. The minimum significance level was $p<0.05$. Principal component analysis (PCA) was employed to detect variables that significantly contributed to differences between the localities. Post-hoc pair-wise comparisons were performed using the Spearman rank order correlation between investigated parameters and localities to determine which values differed significantly. The statistical protocols described by Darlington et al. (1973) and Dinneen and Blakesley (1973) were followed.

RESULTS

Water and air temperatures (C°) at both investigated localities, as well as SVL (cm) and body weights (g) of the snakes are presented in Table 1.

Examination of the activities of antioxidant enzymes revealed that CAT (Fig. 2b), GSH-Px (Fig. 2c) and GR (Fig. 2d) activities in RBCs were significantly lower in *N. tessellata* from the contaminated area than from control area ($p<0.05$). At the same time, the activity of CuZn SOD (Fig. 2a) was not significantly different between animals from the two localities.

The activity of the biotransformation phase II enzyme GST (Fig. 3a) and the concentration of total GSH (Fig. 3b) were significantly higher ($p< 0.05$) in the plasma of snakes from the contaminated area as compared to snakes from the control area. The concentration of lipid peroxides, expressed as the blood TBARS level (Fig. 3c), and the activity of plasma AChE (Fig. 3d) were significantly lower in snakes

Table 1. Water and air temperatures (C°) at the investigated localities (Obedska Bara and Pančevački Rit) at the day of sampling, and the means ± SE of SVL (snout-vent length in cm) and body mass (g) of dice snakes.

	Water temperature (C°)	**Air** temperature (C°)	**SVL** (cm)	**Body mass** (g)
Obedska Bara	18	22.2	67.7 ± 4.26	131.7 ± 30.77
Pančevački Rit	16.5	24.7	60.2 ± 0.90	77.9 ± 7.91

Table 2. Spearman rank order correlations in the blood of dice snake (*Natrix tessellata*) between investigated localities. Marked correlations are significant at *p<0.05, **p<0.01, ***p<0.001.

	Spearman R	***p*-level**
Locality & CuZn SOD	0.11	0.680
Locality & CAT	0.84*	0.001***
Locality & GSH-Px	0.73*	0.001***
Locality & GR	0.81*	0.001***
Locality & GST	-0.55*	0.031*
Locality & GSH	-0.67*	0.004**
Locality & TBARS	0.86*	0.019*
Locality & AChE	0.56*	0.000***

from contaminated area compared to those from control area (p<0.05).

Spearman rank order correlations (Table 2) between the investigated parameters and localities confirmed the results of non-parametric statistics. They show significant correlations between all of the examined parameters, except for CuZn SOD (correlations are significant at p<0.05).

Principal component analysis (PCA) of all of the investigated parameters is presented in Fig. 4. It showed that Principal component 1 and Principal component 2 are responsible for more than 70% of the total variance in the data matrix. PCA 1 explains 46.66% of the total variance, and is mainly characterized by negative loading of the variables GST and GSH, and positive loading of all other investigated variables. PCA 2 explains 27.43% of the total variance and is mainly characterized by negative loading of the variable GST, GSH-Px and GR, and positive loading of all other investigated variables.

The concentrations of 25 trace metals were determined in the water from the two localities (Table 3). Ag, Bi, Cd, Co, Hg, In and Tl were not detected in either locality. Cr, Mo and Pb were not detected at the control area but were present in the water from the contaminated area. The concentrations of Sr were similar in both localities. The concentration of Mg was 2-fold higher in the control area than in contaminated area. The concentrations of Al, As, B, Ba, Ca, Cu, Fe, K, Li, Mn, Na, Ni and Zn were significantly higher in the water from the contaminated area compared to the control area. The most pronounced differences were observed for Al, Fe, K, Li, Mn, Ni and Zn. In the EU, eleven heavy metals of concern are As, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, Sn, and Tl (Kapustová, 2009). Of these, Cd, Co, Hg and Tl were not detected at either locality, while the concentrations of As, Cu, Mn and Ni were increased in the water from the contaminated area compared to the control area. Pb and Cr were not detected in the control area.

DISCUSSION

Studies of reptilian toxicology have mostly focused on organic contaminants, while studies devoted to the effects of metals are scarce (Perault et al., 2013), although many papers have examined the bioaccu-

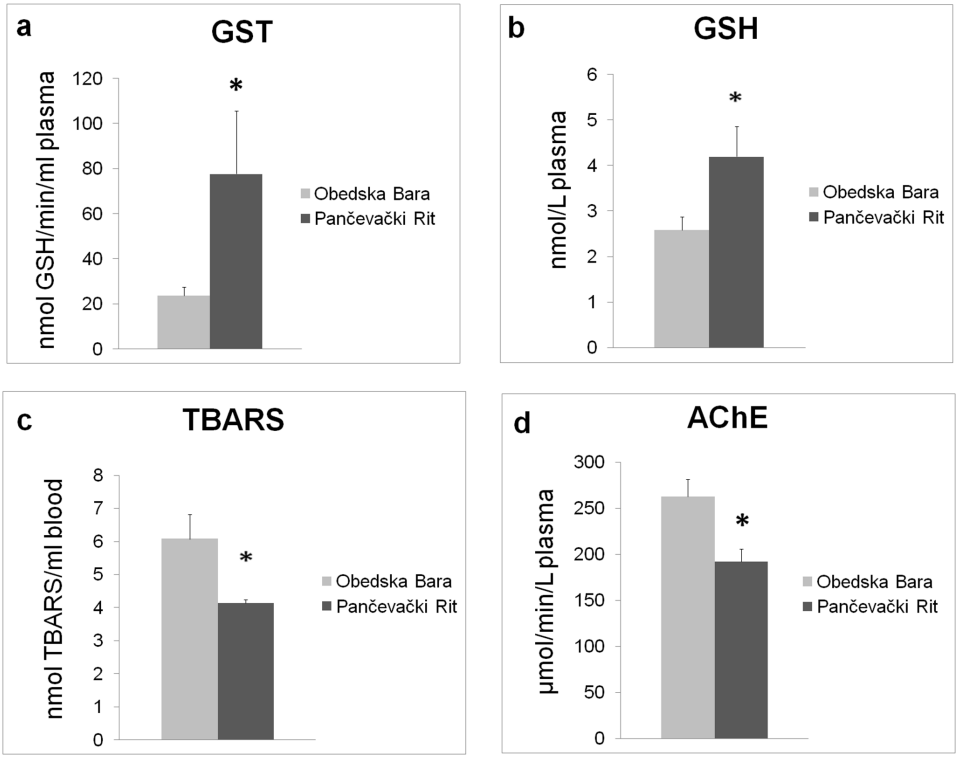

Fig. 3. Changes in different parameters in snakes in response to metal contamination. **A** – the activity of the plasma phase II biotransformation enzyme glutathione-S-transferase (GST, U mL plasma^{-1}); **b** – plasma concentration of total glutathione (GSH, nmol L plasma^{-1}); **c** – blood concentration of lipid peroxides (TBARS, nmol L blood^{-1}); **d** – plasma acetylcholinesterase activity (AChE, U L plasma^{-1}) in the dice snake (*Natrix tessellata*) from the Obedska Bara and Pančevački Rit localities. The samples were compared using the Mann-Whitney *U*-test; (*) significant difference (p<0.05). The number of different specimens used to calculate the means are: N=10 for Obedska Bara and N=7 for Pančevački Rit.

mulation of metals in tissues of various species in field conditions (Yu et al., 2011; Marques et al., 2011). Since the bioaccumulation of metals in the tissues of reptiles has been established, the present study focused on oxidative stress parameters, biotransformation and neurotoxicity biomarkers in order to assess the relationship of the cellular response in the blood of *N. tessellata*, to the presence of metals at two sites with different levels of metal contamination. In the natural environment, this response is more complex due to complicated effects between environmental factors.

While oxygen is necessary for aerobic life, it is also potentially toxic due to the process of biotransformation to reactive oxygen species (ROS). ROS include many potent oxidizing agents, such as superoxide anion radicals, hydrogen peroxide, singlet oxygen, hydroxyl radicals and others. ROS react with biomolecules in the cells, leading to lipid peroxidation, damage of membrane proteins which disturbs the permeability of cell membranes, damage to lysosomes, accumulation of Ca^{2+} ions, disruption of signal transduction, mitochondrial and DNA damage (Halliwell and Gutteridge, 2007). ROS production is potentiated by many environmental contaminants, such as metals, polycyclic aromatic hydrocarbons (PCBs), polychlorinated biphenyls (PAHs), dioxins, etc. (Van der Oost et al., 2003). Hibernating animals, such as the dice snake, have developed mechanisms that oppose oxidative stress during hibernation and/or estivation in the course of evolution (Hermes-Lima and Zenteno-Savín, 2002). Metals can induce oxidative damage direct-

Table 3. Concentrations of dissolved metals in water (μg/L) of the Obedska Bara and Pančevački Rit localities. The mean values ± SE were obtained from three independent measurements.

	Locality		Analytical parameter of method
Element	**Obedska bara**	**Pančevački Rit**	**Detection limit**
Ag	n.d.	n.d.	0.471
Al	581.54 ± 9.24	13276.40± 140.26	3.091
As	22.50 ± 2.15	29.01 ± 1.89	6.666
B	158.45 ± 0.90	302.90 ± 2.30	0.226
Ba	112.64 ± 0.58	198.28 ± 1.7	0.016
Bi	n.d.	n.d.	4.638
Ca	129508.77 ± 897.76	177432.10 ± 8195.31	1.721
Cd	n.d.	n.d.	0.238
Co	n.d.	n.d.	0.829
Cr	n.d.	10.05 ± 0.04	0.490
Cu	15.34 ± 0.32	49.28 ± 0.98	10.458
Fe	619.15 ± 5.25	18193.30 ± 88.96	13.234
Hg	n.d.	n.d.	2.211
In	n.d.	n.d.	3.669
K	4221.62 ± 30.86	20768.08 ± 195.91	6.434
Li	2.55 ± 0.01	15.07 ± 0.07	2.771
Mg	53521.53 ± 202.07	25788.33 ± 152.78	5.753
Mn	77.42 ± 0.63	1425.36 ± 6.09	7.305
Mo	n.d.	3.99 ± 0.20	6.573
Na	43866.27 ± 213.13	52836.27 ± 8.22	6.593
Ni	2.70 ± 0.07	26.38 ± 0.13	11.352
Pb	n.d.	80.50 ± 0.43	2.996
Sr	280.21 ± 0.79	275.08 ± 1.86	3.325
Tl	n.d.	n.d.	1.947
Zn	50.40 ± 0.16	225.04 ± 2.33	0.362

ly by increasing the cellular levels of ROS and by reducing cellular antioxidant capacity (Pinto et al., 2003). Iron, copper, chromium and vanadium undergo redox cycling, while cadmium, mercury, and nickel, as well as lead, deplete the levels of GSH and protein-bound sulfhydryl groups (Stohs and Bagchi, 1995). Metals have high affinity for thiol groups in enzymes, and long-term exposure to these agents could lead to apoptosis as a result of metal-induced cell dysfunction (Flora et al., 2008). Metals can either increase or decrease hepatic enzyme activities and can lead to histopathological hepatic changes (Deore and Wagh, 2012). The toxicity of metals depends on their solubility, absorption rate, transport, chemical reactivity, and the ability to form metallothioneins within the body (Paris-Palacios et al., 2000). Metal contamination in water can directly affect the oxidative status in water snakes, but it can also indirectly induce oxidative stress via the food chain after consumption of contaminated fish. To

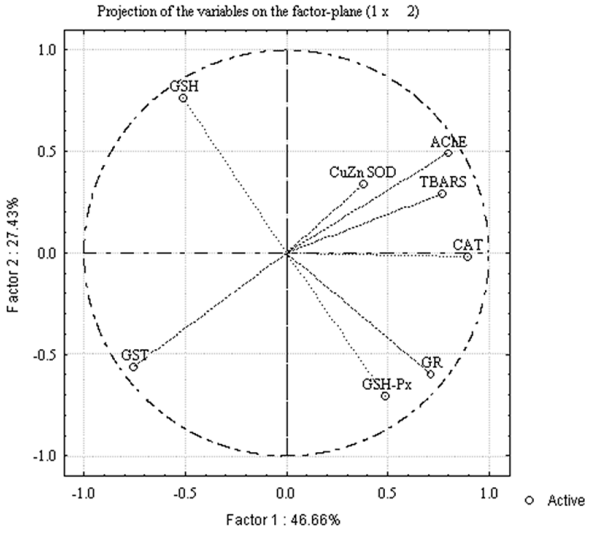

Fig. 4. Principal component analysis (PCA) based on correlations. Projection of all investigated parameters: oxidative stress biomarkers (CuZn SOD, CAT, GSH-Px, GR, GSH, TBARS), biotransformation phase II enzyme (GST) and acetylcholinesterase activity (AChE) on the factor plane.

properly evaluate the influence of metals on aquatic organisms, chemical analyses of metal concentrations need to be supplemented by assessments of its biological effects (Nigro et al., 2006).

The position of snakes in the food chain makes them very suitable for studying the accumulation of contaminants and biomonitoring. Snakes play important ecological roles in controlling the flow of nutrients, energy and contaminants in food webs. According to Hopkins et al. (1999), studies of environmental contamination are available for the family Colubridae, while the most studied species are the water and salt-marsh snakes from North America (*Nerodia spp*). Studies of the water snake *Nerodia fasciata* showed that this species is susceptible to the bioaccumulation of environmental contaminants, suggesting that they can serve as bioindicators of metal contamination (Hopkins et al., 1999). According to Stafford et al. (1976), snakes with high levels of detoxifying enzymes, particularly microsomal oxidases, are more likely to occur in contaminated ecosystems than snakes whose detoxifying enzymes are less active. It was shown that ectotherms that have a low metabolic rate and relatively simple enzyme systems have little ability to detoxify inhaled or ingested pesticides (Lambert, 1997).

There are no established baseline measurements for a wide variety of blood biochemical parameters in snakes that can be used as reference values to describe a specific physiological condition. Hematological studies on snake species have been limited to studies of the morphology and size of red and white blood cells (Arikan et al., 2009), and a limited number of parameters, such as the hematocrit, hemoglobin concentration and standard biochemical indicators (Čož-Rakovac et al., 2011; Tosonoğlu et al., 2011).

Depending on the type of organism, seasonal fluctuations, lengths of exposure, concentrations and interplay of different contaminants, environmental factors can elicit either increases or decreases in antioxidant enzyme activity (Van der Oost et al., 2003). The enzymes SOD and CAT are the first line of defense against free radicals (Halliwell and Gutteridge, 2007). In the present study, we did not observe any statistically relevant differences in CuZnSOD activities between sites, while CAT activity was significantly lower in snakes from Pančevački Rit compared to snakes from Obedska Bara. Borković-Mitić et al. (2013) described a significant negative correlation between CAT activity and the concentrations of Cu, Ni and As. The results of our study are also in agreement with Tsangaris et al. (2010) who studied mussels. These authors demonstrated that CAT activity generally was lower in caged mussels at sites influenced by anthropogenic activities when compared to reference sites. Lead alters cellular calcium metabolism, it is responsible for ROS generation (Al-Kahtani, 2009) and exerts inhibitory effects on both SOD and CAT activities (Borković-Mitić et al., 2013). In contrast to this finding, SOD and CAT activities in the mussel *Mytilus galloprovincialis* were observed to increase at least 2-fold and 2-3-fold, respectively, at contaminated sites when compared to the control site (Vlahogianni et al., 2007). Generally, the course and degree of change in enzymatic activities depends on the types of organisms, doses of contaminants

and the length of exposure. Brucka-Jastrzębska and Kawczuga (2011) showed that elevated metal concentrations increased lipid peroxidation in the blood and dorsal muscle of three fish species and simultaneously reduced SOD activity. Acute exposure to Cd leads to decreased activity of SOD in goldfish erythrocytes (Žikić et al., 2001).

The activity of GSH-Px in the blood of snakes from the contaminated site Pančevački Rit was significantly lower than in snakes from the control site Obedska Bara. This result coincided with the decreased TBARS concentration in the blood of snakes from Pančevački Rit. Lipid peroxides in the blood are recognized as good markers for biomonitoring. They are an important outcome of polyunsaturated fatty acid oxidation during oxidative stress (Ognjanović et al., 2008). Pavlović et al. (2001) found that chronic exposure to heavy metals causes an adaptive response, manifesting as decreased TBARS concentration in the blood. The snakes from Pančevački Rit were persistently exposed to heavy metals in the water. Consequently, the TBARS concentration in their blood was decreased. Higher concentrations of organic hydroperoxides lead to elevated GSH-Px activity, increased utilization of NADPH and production of the oxidized form, $NADP^+$. In order to regenerate the levels of reduced NADPH, the increased utilization of reduced GSH; this influences increased GR activity to revert GSSG to GSH and maintain sufficient amounts of reduced equivalents in cells, thereby normalizing redox homeostasis (Pavlović et al., 2010). Decreased activity of GSH-Px and TBARS concentration reverse the course of these processes.

The phase II biotransformation enzyme (GST) is a reliable biomarker of both inorganic and organic contamination (Van der Oost et al., 2003). In our experiments, we measured elevated GST activity in the blood of snakes from Pančevački Rit compared to specimens from Obedska Bara. As in many other aquatic organisms, the induction of plasma GST activity could be considered as an early-warning and sensitive biomarker of metal exposure in snakes. According to Aguilera et al. (2012), GST and SOD are associated with oxidative stress and processes of detoxification. They generally point to pollution caused by heavy metals and/or hydrocarbons, which are common contaminants in industrial sites.

Since the 1970s, inhibition of AChE activity has been widely used as a specific biomarker for exposure of aquatic species to organophosphate and carbamate pesticides (Fulton and Key, 2001). More recently, studies have shown that high levels of heavy metals depress AChE activity (Vidal-Liñán et al., 2014). Frasco et al. (2006) showed that copper, zinc, cadmium and mercury inhibit AChE activity *in vitro*. AChE inhibition leads to overstimulation of the central and peripheral nervous systems, resulting in neurotoxic effects and consequently death. Inhibition of AChE activity also leads to behavioral changes and impairments at the population level (Xuereb et al., 2009). Our results reveal the inhibitory effects of heavy metals on AChE activity in the blood of snakes from Pančevački Rit when compared to snakes from Obedska Bara. According to other authors (Aguilera et al., 2012; Frasco et al., 2006), GST activity can be a useful biomarker of long-term exposure, whereas AChE activity may be more indicative of acute exposure to contaminants.

To confirm the influence of site on the investigated parameters, we performed Spearman rank order correlations, and obtained significant differences for all of the investigated parameters, except for CuZn-SOD. The PCA referred to the relative contribution of every parameter in the blood. It showed that Factor 1 and Factor 2 explain over 70% of the total variance in the data matrix. The greatest contribution to total variance was due to CAT and AChE activities for Factor 1, and GSH concentration and GSH-Px activity for Factor 2.

CONCLUSION

Differences in investigated parameters were observed in the blood of snakes from different localities: the control locality at Obedska Bara and the contaminated locality at Pančevački Rit which displayed different qualitative and quantitative profiles

of metals dissolved in water. The observed changes indicate that the biomarkers of oxidative stress (CAT, GSH-Px, GSH, TBARS), GST, a phase II biotransformation biomarker, and AChE, a biomarker of neurotoxicity, provide useful data for the evaluation of metal contamination in aquatic ecosystems, since the changes in the above parameters correlated with metal concentrations in the water. The sensitivity of the dice snake to exposure to metals in the environment renders them a sensitive and valuable bioindicator species for ecotoxicological studies.

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Conflict of interest disclosure

The contents of the manuscript have not been published previously, or have been submitted elsewhere for consideration, nor are they in press. All of the authors have seen and approved the manuscript. There is no conflict of interest, including any financial, personal or other relationships with other people or organizations.

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