Carpathian Journal of Earth and Environmental Sciences, August 2021, Vol. 16, No. 2, p. 463 – 468; DOI:10.26471/cjees/2021/016/191

MONITORING OF ENVIRONMENTAL POLLUTION BY HEAVY METAL THROUGH THE ROMAN SNAIL (*HELIX POMATIA*) IN MITROVICA – KOSOVO

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Abstract - The toxic metals biomonitoring of the environment around the metallurgical complex of "Trepça" in Mitrovica, Kosovo were realised. Our investigation has taken place in Mitrovica (area with heavy metal pollution) and Vernica; Vushtrri (control group), 25 km far from the contaminated area. For this purpose, we have analyzed 22 samples of Roman snails (*Helix pomatia*) in each area to determine the amount of total proteins (TP) in the hemolymph and hepatopancreas; heavy metals (Pb, Cd, Cu, Zn) concentration and the activity of delta-aminolevulinic acid dehydratase enzyme (ALAD) in hepatopancreas. Our results show a significantly lower amount of total proteins in the Mitrovica (Mitrovica:16.16±2.419; Vernica:17.9±9.22; P< 0.013). The concentration of metals (Mitrovica: Pb=37.6 ± 2.1, Cd=605.2 ± 308, Zn=51.7 ± 27.8 and Cu=8.5 ± 6.3; Vernica: Pb = 3.7 ± 3.8, Cd=49.22 ± 25.7, Zn=3.9 ± 2.8 and Cu=7.3 ± 6.8) were significantly higher (P < 0.001). We found out the significantly positive correlation between all metals in the study: Pb/Cd (r=0.572; P<0.005); Pb/Cu (r=0.758; P<0.001); Pb/Zn (r=0.857; P<0.001); Cd/Cu (r=0.693; P<0.001); Cd/Zn (r=1.00; P<0.001); Cu/Zn (r=0.685; P<0.001). The activity of ALAD was significantly inhibited (Mitrovica=3.25 ± 1.25; Vernica=6.4 ± 2.3; P<0.001) and the correlation between ALAD and Pb was significantly negative (r=-0.472; P=0.026). The polluted environment with heavy metals in Mitrovica (mostly with the Pb and Cd) harms the biochemistry of Roman snail. This animal species may be used as a good animal model to monitor environmental pollution with heavy metals.

Keywords: Total proteins, hemolymph, hepatopancreas, delta-aminolevulinic acid dehydratase, heavy metal.

1. INTRODUCTION

If we take into account the importance of the environment for the normal development of living beings in general and human health in particular, the situation is very worrying. Directly or indirectly, the extraordinary development of technology and the great desire of people to make money without sparing the destruction of the environment, have led to great environmental disorders. As a consequence of this, many diseases that are often fatal to humans and other living beings have appeared.

Heavy metals, in the form of their compounds, as salts, or gases that are released in the environment from the industrial refineries, mining, heavy metals (HMs) containing wastewater and the traffic, enter the organisms through the respiratory and digestive system and the skin, and in general, (some of them less and others more) represent serious risks to the health of the living beings, such as negative effects in the morphology, structure (histology) and physiology of tissue and organs (Milaimi et al., 2016a, Milaimi et al., 2016b, WHO, 1995). Due to their ability to accumulate in the organisms (due to their atomic weight), and their inability to be metabolized by the organisms, HMs can turn into even more toxic substances for organisms than their original compounds.

Lead is one of the metals that is founded everywhere in nature. In the environment, it is widely released from the different anthropogenic activities and has very toxic abilities at the above-recommended level. Exposing of organisms in the HMs that are released from industrial wastes even though in low doses has brought up an increase of their affinity for exhibiting adverse effects, such as the risk of chronic toxicity, mutagenic, teratogenic, carcinogenic effects on humans and other living organisms. It is documented that in the very low concentration, Pb was an inhibitor of ALAD enzyme activity (Milaimi et al., 2015, Krasniqi et al., 2020), and the degree of inhibition of ALAD in the erythrocytes of human and animal studies, is used as a biological index to the Pb exposure.

There is a wide range of scientific works which have oriented us to use the roman snail as an indicator organism for the HMs pollution. Due to its lightweight; large reproductive capacity; its metabolism; ubiquity; behaviour and presence in large numbers even with the high HMs concentration in the environment, molluscs may be suitable to be used as bioindicator organisms for the HMs contamination. According to the various authors (Coughtrey et al., 1977, Beeby & Richmond 2002), molluscs can accumulate heavy metals in their soft tissues and shells. Because of such accumulation of HMs (Pb, Cd, Zn, Cu), it can result in the inhibition of the protein synthesis in the hepatopancreas (El-Khayat 2016), as well as inhibition of ALAD activity. The purpose of our study was to monitor the environmental pollution with heavy metals and to determine whether the Mitrovica city is still a region with high HMs pollution. This would lead us to discover how these HMs affect the chemistry of the Roman snail and if it is appropriate (*Helix pomatia*) to be used as a bioindicator organism for heavy metal pollution.

2. STUDY AREA

Environmental pollution in Mitrovica city and its surrounding area has reached great proportions after 1930 when the exploitation of the Trepça mine was made by the English company "Trepça mines Limited". In the past, the main sources of industrial pollution have resulted from the various technological units (smelter, refinery, flotation, accumulator factory and sulfuric acid factory) of the "Trepça" plant and the superphosphate fertilizer factory. The main pollutants in the atmosphere were: sulfur dioxide (SO_2), fluorine, lead and cadmium, which exceed the maximum permissible concentration by tens and sometimes by hundreds of times (Graziano et al., 1990, Popovac et al., 1982). The lead smelter was one of the largest smelters in Europe and also was the main pillar of Kosovo's economy, but, at the same time, created a large-scale risk to human health, as a result of environmental pollution from various substances released during the metal processing. According to the authors (Popovac et al., 1982), the average value of the lead concentration in the air of this region was ranged from 7.8 to 21.7 $\mu g/m^3$ in 1973. Meanwhile, in 1980, the concentration of lead in the air was increased from 21.3 to 29.2 $\mu g/m^3$. Although today the smelter "Trepça" is not active, the amount of lead in the environment continues to be at least 2 times higher than the standards recommended by the European Commission (Krasniqi et al., 2020).

3. MATERIAL AND METHODS

Our investigation was realized in two locations: In Mitrovica (contaminated area) and in Vernicë village (Vushtrri) 25 km far away from Mitrovica, in which case 22 samples of the Roman snails (*Helix pomatia*) (approximately in the same size) were analyzed to determine the amount of total proteins (TP) in hemolymph and the hepatopancreas, heavy metals (Pb, Cd, Cu, Zn) concentration and the activity of delta-aminolevulinic acid dehydratase enzyme (ALAD) in the hepatopancreas. After sampling, the snails were placed into the plastic bags and were transported to the laboratory which was kept at room temperature. The hemolymph of the Roman snail was taken without anaesthesia using a syringe on the leg muscles. Before it was used, the hemolymph was kept without anticoagulant at the - 20°C. The amount of total proteins was performed by the spectrophotometric method according to the Lowry method. During the experiment, ethical guidelines were followed where we tried to take a few samples as possible. The determination of the activity of the ALAD enzyme was done by the method of Bonsignore (Bonsignore et al., 1965). For each sample were weighed 200 mg of hepatopancreatic tissue and homogenized with 2.6 ml cold phosphate buffer at pH 6.4. The homogenate was previously placed in the test tube with the ice water; wrapped with the aluminium foil and then was centrifuged at 4000 rpm / 5 minutes. Into the other three test tubes were added 0.4 ml of supernatant and 2.6 ml of phosphate buffer at pH 6.4 and were mixed well and placed into the water bath for 10' / 37ºC. After 10 minutes into the blank test tube were added 2 ml of 10% trichloroacetic acid (TCA) solution and to all three test tubes were added 2 ml of 0.01 M aminolevulinic acid substrate prepared with phosphate buffer at pH 6.4. All the test tubes were placed in the water bath at 37ºC/60'. After this, we were added 2 ml of 10% TCA solution and the test tubes were centrifuged at 3000 rpm / 10'. 2 ml of pure supernatant were transferred into the other clean and darkened test tubes. Then, were added 2 ml of fresh Ehrlich reagent. After 5 minutes we read the absorbance in the spectrophotometer, in the wavelength of 555 nm compared to the H_2O-Re. Enzyme activity was expressed in µmol porphobilinogen synthesized per mg/g of proteins within one hour at 37ºC (µmol PBG/mg/g protein/h). The heavy metals concentration (Pb, Cd, Zn and Cu) has analyzed in the homogenized hepatopancreas tissue. The hepatopancreas samples were weighed and were dried in thermostat at 75˚C for 48h. The samples were ground and were weighed 0.3g of each of them. Then, the samples were digested with royal water (7ml HNO_3 and 3ml H_2O_2) in the microwave. The determination of the total content of heavy metals was done with the ICP-OES apparatus, Optima 2100 DV (Perkin Elmer Optima 2100 DV ICP - OES). Metal detection levels were: for Cd: 0.1ppb, Cu: 0.4ppb, Pb: 1ppb, and Zn: 0.2ppb. The result is given as µg/g. Statistical analyzes were performed using the statistical program (Sigma stat 3.5). We have calculated the arithmetic mean, standard deviation, significance and correlation (Pearson correlation) between the metal concentrations and between lead and the ALAD activity in the hepatopancreas tissue.

4. RESULTS AND DISCUSSION

The amount of total proteins (Table 1) in the hemolymph of snails in the Mitrovica group (65.4 ± 7.46 µg / ml) compared to the control group (84.2 ± 8.2 µg / ml) were significantly lower (P <0.001) in the hemolymph and the hepatopancreas (Mitrovica: 78.2 ± 14.6 µg / g; Control: 134.2 ± 12.7 µg / g.

All of the HMs depending on the type of their compounds were found in the environment as a result of the anthropogenic activities. So, in our case, HMs (Pb, Cd, Cu and Zn) mainly were derived from the lead metallurgy ("Trepça" smelter) in Mitrovica - Kosovo. HMS tend to be accumulated in the target organs and transferring to humans through the food chain (Carbone 2009). It has been founded that the largest amounts of Pb, Cu and Zn have been accumulated in the bones (about 90%), whereas the Cd in the liver and kidneys (Milaimi et al., 2016a, Genchi et al., 2020) of the animal models and humans exposed to the HMs in the industrial areas.

As a result, HMs prompt many defects of cell functions in the liver tissue. (Levina 2021, Ciosek 2021). Pb and Cd were accumulated in the liver tissue of feral pigeon (Milaimi et al., 2016a, Krasniqi et al., 2020) and albino rats (Metwally et al., 2015) exposed naturally to these metals and have inhibited the protein synthesis, and also impaired the enzymatic activities (Krasniqi 2020). Such as the liver, the hepatopancreas is considered the main organ of metabolic functions and organism detoxification of snails. The proteins that are present in the hemolymph (enzymes, hormones, many lipoproteins and glycoproteins) are synthesized mainly in the hepatopancreas and have different natures and functions, so any decreases in their level can be taken as a measurement parameter for the hepatopancreas function (Bislimi et al., 2015). So, the adverse effects of Pb and Cd have also been found in the hepatopancreas of the snails' *Helix aspersa* (Carbone & Faggio 2009). The Pb and Cd have promoted lipid peroxidation leading to oxidative stress and resulting in modification of protein content in the liver of vertebrates (Metwally et al., 2015). In the hepatopancreas of *Helix pomatia,* has been reported for the high level of oxidative stress enzymes and lipid peroxidation end products as a result of Cd, Pb and Zn accumulation (Nowakowska et al., 2014). Concentration of HMs (Pb, Zn, Cd and Cu) in the hepatopancreas (Table 2) were significantly higher (P <0.001) for all metals in the Mitrovica group (Pb=37.6 ± 2.1; Zn=605.2 ± 308; Cd=51.7 ± 27.8; Cu=8.5 ± 6.3) compared to the control group (Pb=3.7 ± 3.8; Zn=49.22± 25.7; Cd=3.9 ± 2.8; Cu=7.3 ± 6.8). In addition to that, due to the high accumulation of all metals (Pb, Cd, Zn and Cu) in the hepatopancreas of roman snails in the Mitrovica group may have resulted in the dysfunction of the hepatopancreas leading to the inhibition of the protein synthesis.

Authors who have studied the accumulation of heavy metals in the hepatopancreas tissue of *Helix pomatia* in the HMs pollution areas (Ziomek et al., 2018, Nica et al., 2012) have found out the major concentration of Zn, Pb and Cd (Zn:387, Pb:1.96, Cd:1.43 mg/kg^{-1}) in the hepatopancreas compared to

Table 1. Mean amount of total proteins (TP) in the hemolymph and hepatopancreas of the Roman snail (*Helix pomatia L.*) in the study area and control group.

LOCALITY	TP in hemolymph µg/ml	TP in hepatopancreas µg/g
Vernica	84.2 ± 8.2 (21)	134.2 ± 12.7 (21)
Mitrovica	65.4 ± 7.46 (21)	78.2 ± 14.6 (21)
Significant: P <	0.001	0.001

Note: the above results were represented as arithmetic mean X; standard deviation: ±; P - significance; in parentheses the number of individuals.

Table 2. The concentration of the heavy metals (mg/kg of dry weight) in the hepatopancreas of Romas snail (*Helix Pomatia*) in the study area and control group.

LOCALITY	Pb	Zn	Cd	Cu
Mitrovica	37.6 ± 2.1 (22)	605.2 ± 308 (22)	51.7 ± 27.8 (22)	8.5 ± 6.3 (22)
Vernica	3.7 ± 3.8 (22)	49.22± 25.7 (22)	3.9 ± 2.8 (22)	7.3 ± 6.8 (22)
Significant: P <	0.001	0.001	0.001	0.001

Note: the above results were represented as arithmetic mean X; standard deviation: ±; P - significance; in parentheses the number of individuals.

the other tissues, so the hepatopancreas is macro concentrator for the Pb, Cd and Cu, and accumulate much more metals compared to the other snail's tissue (Vukašinovi´c-Peši´c et al., 2020, Mahmutovic et al., 2018). Consequently, therefore, we may reason the disfunction of hepatopancreatic tissue as e result of higher concentration of metals in Mitrovica (Pb: 37.6, Zn: 605.2, Cd: 51.7, Cu: 8.5 mg/kg).

Accumulation of lead in the hepatopancreas (table 3) was associated with an inhibition of ALAD activity of the Mitrovica group (ALAD: 3.25 ± 1.25) compared to the control group (ALAD 6.4 ± 2.3) at a significant degree P <0.001. The Pearson correlation between the metals in the hepatopancreas of snail's form Mitrovica were significantly (P <0.001) positive (r=; Pb/Cd=0.572; Pb/Cu=0.758; Pb/Zn=0.857; Cd/Cu=0.693; Cd/Zn=1.00; Cu/Zn=0.685) (table 4.) and the correlation between the lead concentration and ALAD activity was negative (r-0.472) in the significance degree P <0.001.

δ-aminolevulinic acid dehydratase is one of the enzymes that are zinc-dependent and is inhibited by the presence of lead. It is thought that four zinc atoms are needed to prevent the oxidation of sulfhydryl groups on the active site of the ALAD enzyme, while four more atoms are needed to maintain the stability of the homoctameric structure of the enzyme. Lead cations can transpose the zinc cations to the active site of the enzyme and this inhibition is reversible in vitro conditions (Wetmur et al., 1991). Cd is the co-contaminant metal with the Pb and they do not have any biological function. Zn and Cu, on the other hand, have the function as a cofactor, or as a structural component of the many enzymes, non-enzymatic proteins, and therefore play a role in a wide range of biological processes (Sloup et al., 2017, Hefnawy 2015). Furthermore, substituting zinc with the lead in the zinc-dependent enzymes, or the other proteins, may inhibit the enzymes/proteins function (Ugwuja et al., 2020).

Inhibition of ALAD activity has been used as a biomarker in the detection of lead exposure in animals. In the wild population of birds, the Pb is a 10 to 1000 times potent inhibitor of ALAD activity compared to the Cu, Cd, or Hg, but emphasizes the possibility that both, Cd and Cu have inhibited ALAD activity, also (Anant et al., 2018).

Our results about the inhibition of ALAD activity and negative correlation between the ALAD activity and Pb concentration (Table 4) are in agreement with the results of the other authors (Krasniqi et al., 2020, Scheuhammer 1987), those who in the Mitrovica found out the high inhibition (Pb in blood: 0.040±0.027; ALAD: 3.7±1.3: P <0.001; r=-0.811; P <0.001) of the ALAD activity in the blood of grazing cows from "Trepça" smelter (Kelmend) compared to the other groups far away from Kelmend, respectively in the wild population of *Columba livia* (Pb: 17.1±10.3 µg/dL; ALAD: 17.6 ± 12.6; P<0.001; r = - 0.409; P<0.05). Referring to these data, in Mitrovica city, the snails accumulated a higher concentration of Pb in the hepatopancreas compare to Feral pigeon and the Cows from the same area (Kelmend – 2 km far away from the "Trepça" smelter). The latest data (Memishi et al., 2020) showed that in Kelmend (Mitrovica), the concentration of the lead in the environment is higher than the permissible limits from BE (soil: 184 ± 114 mg/kg^{-1}, grass: 37.0 ± 10 mg/kg^{-1}, cow blood: 0.040 ± 0.027 mg/kg^{-1}; ALAD activity: 3.7 ± 1.3; P <0.001). This condition is very significant for us, which proves to us that the area where the snail samples were fed is very contaminated with lead metal.

The Zn and Cu metals are essential for organisms, but, high concentrations can also lead to problems in the living being organs. Chronic poisoning in Zn and Cu, also causes its deposition in various organs (Dehari-Zeka et al., 2020) leading to various histological, biochemical and structural defects (Milaimi et al., 2016b). Toxicity of the animals with

Table 3. The activity of δ -– aminolevulinic acid dehydratase (ALAD) in the hepatopancreas of the Roman snail (*Helix Pomatia*) in the study area and control group.

LOCALITY	ALAD µmol PBG/mg/g prot/h	Pb µg/mg
Mitrovica	3.25 ± 1.25 (22)	37.6 ± 2.1 (22)
Vernica	6.4 ± 2.3 (22)	3.7 ± 3.8 (22)
Significant: P <	0.001	0.001

Note: the above results were represented as the arithmetic mean X; standard deviation: ±; P - significance; in parentheses the number of individuals.

Table 4. The correlation (r) between heavy metals in the hepatopancreas tissue and between lead and ALAD activity of Roman snail in the Mitrovica group.

LOCALITY	HEAVY METALS	CORRELATION (r)	SIGNIFICANCE
MITROVICA	Pb/Cd (22)	0.572	0.005
	Pb/Cu (22)	0.758	<0.001 (0.000)
	Pb/Zn (22)	0.857	<0.001 (0.000)
	Cd/Cu (22)	0.693	<0.001 (0.000)
	Cd/Zn (22)	1.00	<0.001 (0.000)
	Cu/Zn (22)	0.685	<0.001 (0.000)
	ALAD/Pb (22)	-0.472	0.026

Note: the results are expressed as correlation values according to Pearson (r). In parentheses, the number of samples.

the Zn and Cu is encountered very rarely and can be expressed when the organism is subjected to exposure to high doses of Pb and Cd (Anant et al., 2018). According to the results of the other authors (Scheuhammer 1987), high concentrations of the Pb and Cd in the hepatopancreas of snails in the Mitrovica will have brought to the excessive accumulations of Zn and Cu (Zn: 605.2, Cu: 8.5 mg/kg) as a protective effect against the Pb and Cd toxicity.

Recently in Kosovo, the Roman snail is used for human consumption and export. Various snail farms are also located in Mitrovica city. According to the European Commission, 1.5 mg/kg HMs in the foodstuffs (molluscs) is a maximum level for consumption. In our case, the concentrations of the HMs in the hepatopancreas are at much higher values compared to the results of the other authors (Ziomek et al., 2018) who found out the high concentration of heavy metals (Cd=0.19 $\mu g\ g^{-1}$, Cu=11.68 $\mu g\ g^{-1}$, Zn=35.42 $\mu g\ g^{-1}$) in the leg muscle and in the hepatopancreas tissue above the permissible levels for human consumption. The concentration of all metals (especially the Cd) in the hepatopancreas tissue of Roman snail in the Mitrovica, may lead to a high risk for the people eating this kind of food. As a result, the snails from the Mitrovica region may be dangerous for human consumption.

5. CONCLUSION

Our data has shown that in Mitrovica must be taken serious measures to repair further damage to the health of the citizens of this part of Kosovo. Even though the lead smelter does not work nowadays, the Mitrovica city continues to be a contaminated area with Pb, Cd, Zn and Cu which possessing a high risk to the environment and living organisms. Knowing that this effect has long-term consequences, an extended and general analysis of the state of the environment and living beings must be taken into account.

The higher concentration of Pb and the other metals (Cd, Zn and Cu) in the environment; their accumulation in the hepatopancreas of snails in the Mitrovica group has brought to the inhibition of protein synthesis and the ALAD activity.

The Roman snail can be used as a bio-indicator organism to monitor the environmental HMs pollution since it can tolerate and can survive in the high level of heavy metal concentration.

Acknowledgements

This experimental work was done in the University of Prishtina; Faculty of Mathematics and Natural Sciences; Department of Biology; Republic of Kosovo. We are thankful to our colleague: Prof. Dr Kasum Letaj for his assistance with experimental work.

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Received at: 12. 07. 2021
Revised at: 16. 07. 2021
Accepted for publication at: 30. 07. 2021
Published online at: 02. 08. 2021