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Original Research Article

Effects of water contamination with metals on pigmented macrophages in spleen of Vardar chub (*Squalius vardarensis* Karaman, 1928) from three rivers in the North-Eastern region of North Macedonia

LOZENKA IVANOVA[1*]
KATERINA REBOK[1]
MAJA JORDANOVA[1]
ZRINKA DRAGUN[2]
VASIL KOSTOV[3]
SHERIBAN RAMANI[4]
DAMIR VALIĆ[5]
NESRETE KRASNIĆI[2]
VLATKA FILIPOVIĆ MARIJIĆ[2]
DAMIR KAPETANOVIĆ[5]

[1] Institute of Biology, Faculty of Natural Sciences and Mathematics, Ss Cyril and Methodius University in Skopje Arhimedova 3, 1000 Skopje, North Macedonia

[2] Ruđer Bošković Institute, Division for Marine and Environmental Research, Laboratory for Biological Effects of Metals, Bijenička 54, 10002 Zagreb, Croatia

[3] Institute of Animal Sciences, Ile Ilievski 92a, 1000 Skopje, North Macedonia

[4] Ministry of Agriculture, Forestry and Water Economy, Hydrometeorological Administration, Division for Water, Air and Soil Quality Monitoring and Laboratory Analyses; Hydrobiology and Ecology Department, Skupi 28, 1000 Skopje, North Macedonia

[5] Ruđer Bošković Institute, Division for Marine and Environmental Research, Laboratory for Aquaculture and Pathology of Aquatic Organisms, Bijenička 54, 10002 Zagreb, Croatia

***Correspondence:**
Lozenka Ivanova
E-mail address: lozenka@yahoo.com

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Abstract

Background and purpose: *Pigmented macrophage aggregates (PMAs) are a part of the immune system of fish and are considered as valuable biomarkers of the impact of water pollution on fish health.*

Materials and methods: *Immunomodulatory effect of water contaminated with high levels of metals on spleen and splenic PMAs of Vardar chub (*Squalius vardarensis*) (n=129) was examined in two seasons, spring, and autumn 2012. For this purpose, three rivers were chosen: the Zletovska River, heavily contaminated with metals, the Kriva River, moderately contaminated with metals combined with high level of organic matter, and the Bregalnica River, without heavy metal contamination. Spleen masses and splenosomatic indices (SSI) were used for determining the general impact of water contamination on the immune system of Vardar chub. Moreover, volumes and numbers of PMAs within spleen tissue were determined stereologically.*

Results: *The isolated effect of high exposure to metals caused the reduction of spleen mass and/or SSI in the fish from the Zletovska River. The combined effect of exposure to metals and high levels of nutrients in the fish from the Kriva River referred to stimulation of PMAs response, namely increased volumes of splenic PMAs. The existence of seasonally dependent differences was noted since the observed variability in PMAs between rivers occurred only in the autumn.*

Conclusions: *Splenic PMAs in the Vardar chub can be used as an additional biomarker of metal pollution, but with caution, since enhancement*

List of abbreviations:

BM	– body mass
CF	– condition factor
PMAs	– pigmented macrophage aggregates
No.PMAs/mm^2	– number of macrophage aggregates per squared millimeter
SM	– spleen mass
SSI	– splenosomatic index
TL	– total length
Vv(%)	– relative volume of pigmented macrophage aggregates
V(cm^3)	– total volume of pigmented macrophage aggregates
V(spleen)	– volume of spleen
TN	– total nitrogen
TP	– total phosphorus

of the volumes and numbers of PMAs depends on the exposure level of metals, on the presence of the other contaminants, as well as on the physiological condition of the fish.

INTRODUCTION

Rivers in the North-Eastern region of Republic of North Macedonia are under pressure due to the input of different pollutants, including metals from mining activities *(1, 2, 3)*. The three rivers chosen for this study were: the Zletovska River and the Kriva River, which are in proximity of the mines, and the Bregalnica River which is not influenced by the mining, but is influenced by agricultural activities *(1, 2, 3, 4)*. To assess the impact of water quality on living organisms, biomonitoring studies are needed. Fish are considered as good candidates to serve as bioindicator organisms that can be used for monitoring of long-term effects of pollutants as they represent higher trophic levels compared to other water dwelling organisms, and as such are at high risk of bioaccumulation and bioconcentration. One such candidate for the region of North-Eastern Macedonia is the wide-spread fish species, Vardar chub (*Squalius vardarensis* Karaman) which is omnivorous fish that feeds on both plants and smaller animals.

Different pollutants that occur in surface waters, including metals, have been associated with changes in the fish immune system and their general health *(5, 6, 7, 8)*. Spleen is one of the main organs in fish immune defense, and changes in spleen size and structure are considered indicative of negative impact of water pollution *(7, 9, 10, 11)*. Spleen, among other organs such as kidney, liver, gills, and others, is a site of aggregation of pigmented macrophages (PMAs). PMAs represent accumulations of phagocytic cells that belong to the immune system of fish, and their function is centralization, detoxification and recycling of cell debris and foreign bodies *(12, 13)*. As a result of their function, they accumulate various pigments: lipochrome, melanin and hemosiderin, which make them easy to visualize *(12, 13, 14)*. The volume and number of PMAs can change under different conditions, including exposure to pollutants *(8, 15, 16, 17)*. Their phagocytic activity may be affected by various substances that fish are exposed to, as was shown in earlier *in vitro* experiments *(5,18)*. Because they relate to general contamination and are not specific to one type of pollutant, they could be a good additional tool for estimating the general condition of the environment. Therefore, PMAs were proposed as useful biomarkers of river water quality *(8, 12, 13, 14, 19, 20)*. However, seasonal and hormonal changes, which occur during the breeding cycle, can also have effects on occurrence and volume of PMAs *(7, 29, 22, 23)*. Increased PMAs numbers are also connected to other physiological factors like ageing as well as starvation *(24)*. These are some of the factors that can lead to misinterpretation of results, therefore caution and good exploration model needs to be established when using PMAs as biomarkers and which should take into consideration the season, sex, and age.

One of the noticed effects of metals on the immune system is modulation of phagocytic activity of the cells. That influence is considered to be suppressive, but this is still open to debate since some authors reported increased phagocytic activity, which seems to be dose dependent *(18, 25)*, while the others have reported increase of number and volume of PMAs in fish treated with metals *(17, 26, 27, 28)*.

Many of the experiments with metal exposures of fish were carried out with high metal concentrations, which may not be realistic and directly applicable to real world scenarios that are often characterized by chronic low-level exposures. Also, laboratory conditions are not always representative of the more complex interactions in the field *(29)*. Furthermore, modification of fish physiological responses can occur due to the other factors and not only pollution *(9)*, such as food availability and characteristics of their habitat *(29, 30, 31)*. Therefore, the main goal of this investigation was to examine whether and how metal contaminated environment induce changes in body mass, spleen size and PMAs parameters of fish captured in three differently contaminated rivers *(32)*, as well as to examine the possible stimulation and/or inhibition of the physiological responses by the additional presence of organic matter in the water.

MATERIALS AND METHODS

For the purpose of this investigation 129 fish (age 2-5) of the species Vardar chub (*Squalius vardarensis* Karaman, 1928) were collected in two seasons (spring and autumn 2012) from three differently contaminated rivers: the Zletovska River (n=27 for spring, n=12 for autumn season), the Kriva River (n=26 for spring, n=9 for autumn season) and the Bregalnica River (n=26 for spring, n=29 for autumn season). Fish were collected by electro-fishing (electrofisher Samus 725G), according to the CEN EN 14011 standard *(33)*, and complied with the current laws of the Republic of North Macedonia. The information about the water contamination at the time of collection in the three studied rivers was extracted from our previously published paper (32, Table 1). According to these findings, at the times and sites of collection of the fish the Zletovska River had high concentrations of several dissolved metals, with extremely high concentrations of Cd, Zn and Mn in autumn 2012 *(32)*. The Kriva River had generally lower concentrations of dissolved metals compared to the Zletovska River, but it was also burdened with increased concentrations of dissolved Cd and Pb in the spring 2012, probably due to sediment resuspension during the rainy season *(32)*. In addition, organic substances and fecal bacteria were introduced into the Kriva River water due to local agricultural activities and input of municipal waste-

Table 1. *Water quality parameters of three studied Macedonian rivers (Bregalnica, Zletovska, Kriva) in spring and autumn of 2012 at the time and points of fish collection. Results were previously reported by Ramani et al. (32)*

	Bregalnica River		Zletovska River		Kriva River	
	Spring 2012	Autumn 2012	Spring 2012	Autumn 2012	Spring 2012	Autumn 2012
Metals (n=3)						
Cd / µg L^{-1}	0.032±0.008	0.021±0.019	0.272±0.002	2.00±0.03	0.270±0.010	0.029±0.011
Cs / µg L^{-1}	0.067±0.002	0.091±0.002	1.30±0.01	1.03±0.02	0.026±0.001	0.015±0.000
Mn / µg L^{-1}	13.27±0.79	4.40±0.58	351.9±6.5	2527±219	9.90±1.88	9.65±0.96
Pb / µg L^{-1}	0.692±0.096	0.307±0.216	0.313±0.044	0.748±0.099	1.85±0.22	0.420±0.031
Tl / µg L^{-1}	0.014±0.000	0.008±0.001	0.043±0.001	0.146±0.002	0.006±0.000	0.003±0.001
Zn / µg L^{-1}	4.97±1.84	6.14±8.51	197.0±2.9	1507±138	22.07±6.86	3.81±2.85
Nutrients/anions (n=1)						
NH_4^+ / (mg N L^{-1})	0.053	0.096	0.019	0.123	0.360	2.18
TN / (mg N L^{-1})	0.90	1.90	1.50	0.50	0.95	5.10
TP / (mg P L^{-1})	<0.5	0.089	<0.5	0.032	<0.5	0.325
Number of bacteria (n=1)						
Total coliforms (MPN/100 mL)	-	15214	-	3864	-	435170
Enterococci (MPN/100 mL)	-	1005	-	23.5	-	15653

water *(32, 34)*. Bregalnica, on the other hand, had much lower concentrations of dissolved metals, but moderately increased fecal contamination *(32, 34)*. Sites of collection and their proximity to mines are marked on the map downloaded from *www.freeworldmaps.net* (35, Figure 1).

After capture, fish were transported to the laboratory in oxygenated tanks. Shortly before dissection, fish were euthanized with Clove oil (Sigma) and their body masses (BM) and total lengths (TL) were measured. Scales were also collected, and the fish age was determined according

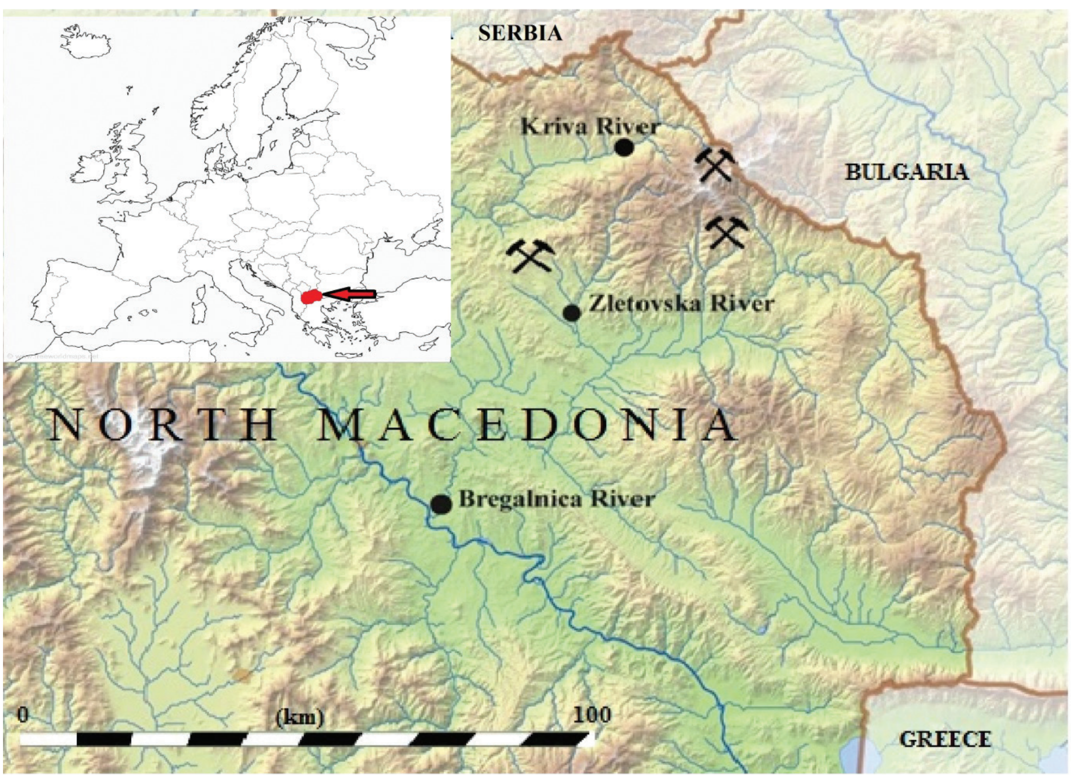

Figure 1. *Maps representing North Macedonia in wider context of Europe (upper left corner) and part of the North Macedonia with marked sites of collection (black circles) and their proximity to mines (right)*

to the annual ring structure of scales. Afterwards, spleens were removed, and spleen masses (SM) were measured. The recorded values of BM and SM were used for calculation of the splenosomatic indices (SSI) according to the following formula (Eq. 1):

$$SSI=SM\times 100/BM \quad (1)$$

Other organs were also collected and investigated by Jordanova et al. *(23, 36)*. Data for gonadosomatic, hepatosomatic and renosomatic indices, from these investigations were used in discussion for context and interpretation of results *(23, 36)*.

Spleens were then fixated in Bouin's fixative for 48 hours, processed in series of alcohols and embedded in paraffin blocks. Paraffin sections (5 µm thin) were cut on manual rotary microtome

Several stains were used for confirmation of the pigments on sections from eight test chub spleen: Masson-Fonatana, Schmorl's and melanin bleaching for confirmation of the presence of melanin pigment, and Sudan black and Zeihl Neelsen for confirmation of lipochrome pigments. For the full sample analysis Perls' stain was chosen as the best method that allows the visualization of the three pigments (melanin, lipochrome, and hemosiderin) in PMAs simultaneously.

Classical stereological method based on point counts *(37)*, was used for estimating relative volumes (*Vv*) and total volumes (*V*) of PMAs. Between three and five sections and at least 50 fields per fish were analyzed, chosen by systematic random sampling (SRS) method. An ocular with a grid of 180 points was used at a magnification of 400×. The counts were used for calculating the *Vv* of PMAs, expressed as %, by the following formula (Eq.2):

$$Vv(\text{PMAs, spleen})= [P(s) \times 100] / P(r) \quad (2)$$

where P(s) was the number of points overlapping with PMAs and P(r) was the number of points overlapping with the parenchyma of the spleen.

Total volumes calculations were made to provide information about PMAs volume changes independent of organ volume changes *(38)* by multiplying *Vv(PMAs, spleen)* to spleen volume *V(spleen)*. The *V* of PMAs in the spleen, expressed as cm^3, was then determined with the following formula (Eq. 3):

$$V(\text{PMAs, spleen}) = Vv\times V(\text{spleen}) \quad (3)$$

The same grid with estimated area of 21,025 $x10^{-3}$ mm^2 based on minimum counts of 50 fields per fish was used for determination of the number of PMAs/mm^2 using the following formula (Eq.4):

$$PMAs/mm^2=No.of\ PMAs/(AF\times 21,025\ x10^{-3}) \quad (4)$$

In order to test the isolated effect of water contaminated with metals, or to test the combined effect of water burdened by both metals and high level of organic matter on fish health, the values of spleen mass (SM), splenosomatic index (SSI) and of three parameters of splenic PMAs of the fish collected from the Zletovska and the Kriva River, respectively, were compared to the values of those same parameters measured for the fish collected from the Bregalnica River. Fish aged under 2 were not considered, as they are considered juvenile. Also, since PMAs parameters values can also increase with age even in normal conditions, to avoid misinterpretation of results as influence of exposure, fish were divided into 2 age groups for the analysis. Results are presented as mean values, followed by the coefficients of variation. Statistical analysis was conducted with Statistica 8.0 for Windows (StatSoft), using two-way ANOVA testing for season and locality, followed by post hoc Newman-Keuls test. Data that were not normally distributed were log10-transformed prior to analysis. Results were considered significant at $p < 0.05$.

RESULTS

Comparing the fish age among localities showed that fish from the Zletovska River collected in autumn were younger (average of 2.5 years) than the fish collected from the other 2 rivers in the same season (average of 3.83 and 3.75 for the Bregalnica and the Kriva River respectively), whereas in the spring season the samples from the three rivers showed no significant difference in age (average of 3.45, 3.05 and 3.31 for the Bregalnica, the Zletovska and the Kriva River respectively). Therefore, we further compared the measured and the calculated values in 2 age groups. The first group consisted of fish aged 2-3 years, and the second group of fish aged 4-5 years old. Results for the second group aged 4-5 for fish from the Zletovska River in autumn were excluded from the additional analysis, because only one fish age 4 was collected. Statistics for BM, TL, and Fulton's condition factor (FCF) were run for comparing fish within the age groups (Table 2). Significantly lower values of SM and SSI were noted for fish age 2-3 years collected from the Zletovska River in the autumn season, when compared to the other two rivers, but was not significant for fish of this age in the spring season (Table 3). Considering the seasons, fish aged 4-5 years captured from the Kriva River in spring also had significantly lower values of SSI compared to the fish of the same age captured in the autumn season (Table 3). Other significant differences were not noted for fish from this age group.

Concerning PMAs, visual inspection revealed that they were randomly distributed within the spleen tissue (Figure 2), and they contained all three pigments in various combinations and ratios. PMAs in spleen of fish from the Bregalnica (Figure 2a) and the Kriva River (Figure

Table 2. *Body masses (BM), total lengths (TL) and Fulton condition factors (Fulton CF) of Vardar chub in two age groups from three differently contaminated rivers, combined for both sexes, and presented separately for each season (spring and autumn)[a]. Data for BM, TL and Fulton's CF for the same Vardar chub specimens were previously reported by Jordanova et al. (36) and Dragun et al. (42), without taking age into consideration.*

	BM (g)				TL (cm)				Fulton CF (%)			
	spring		autumn		spring		autumn		spring		autumn	
Group age 2-3 years												
Bregalnica River	52.25	(24.82)^{p}	74.18	(29.58)^{p}	16.33	(2.55)^{pq}	18.68	(2.38)^{p}	1.12	(0.10)^{p}	1.09	(0.04)^{p}
Zletovska River	22.95	(8.71)^{q*}	17.07	(18.37)^{q}	13.07	(1.56)^{p}	11.44	(3.47)^{q}	0.98	(0.08)^{q}	0.89	(0.06)^{q}
Kriva River	46.64	(11.83)^{p}	57.61	(7.10)^{p}	16.08	(1.34)^{q}	18.10	(1.14)^{p}	1.10	(0.06)^{pq}	0.97	(0.06)^{pq}
Group age 4-5 years												
Bregalnica River	116.,33	(70.,92)^{pq}	91.02	(63.79)	20.70	(3.90)	19.84	(3.97)	1.19	(0.10)P^{p}	1.03	(0.06)^{Q}
Zletovska River	46.11	(26.,46)^{p*}	n/a		16.28	(2.78)	n/a		0.98	(0.07)^{q}	n/a	
Kriva River	115.16	(54.12)^{q}	62.68	(21. 29)	20.82	(3.36)	18.52	(2.01)	1.19	(0.08)P^{p}	0.96	(0.06)^{Q}

[a]Results are presented as mean values, followed by coefficient of variation in brackets. For each value, different lowercase letters in superscript represent significant differences between rivers within the same season (read vertically), different capital letters represent significant differences between seasons within the same locality (read horizontally), and asterisk represents difference among age groups in same season and locality (read vertically), according to two-way ANOVA followed by post-hoc Newman-Keuls test.

Table 3. *Splenosomatic indices (SSI), spleen masses (SM) and age of Vardar chub in two age groups from three differently contaminated rivers, combined for both sexes, and presented separately for each season (spring and autumn)[a]*

	SSI (%)				SM (g)				age (years)			
	spring		autumn		spring		autumn		spring		autumn	
Group age 2-3 years												
Bregalnica River	0.12	(0.05)	0.16	(0.03)^{p}	0.06	(0.02)	0.12	(0.05)^{p}	2.74	(0.45)	2.75	(0.46)
Zletovska River	0.08	(0.02)	0.07	(0.06)^{q}	0.02	(0.01)	0.01	(0.01)^{q}	2.67	(0.48)	2.20	(0.45)
Kriva River	0.13	(0.04)	0.14	(0.01)^{p}	0.06	(0.02)	0.08	(0.01)^{p}	2.69	(0.48)	2.67	(0.58)
Group age 4-5 years												
Bregalnica River	0.10	(0.03)	0.13	(0.04)	0.10	(0.05)	0.12	(0.08)	4.58	(0.51)	4.38	(0.50)
Zletovska River	0.08	(0.02)	n/a		0.04	(0.02)	n/a		4.00	(0.00)	n/a	
Kriva River	0.08	(0.02)^{P}	0.15	(0.01)^{Q}	0.09	(0.04)	0.09	(0.03)	4.30	(0.48)	4.40	(0.55)

[a]Results are presented as mean values, followed by coefficient of variation in brackets. For each value, different lowercase letters represent significant differences between rivers within the same season (read vertically), and different capital letters represent significant differences between seasons within the same locality (read horizontally), according to two-way ANOVA followed by post-hoc Newman-Keuls test.

2b) contained mainly melanin and lipochrome pigments, whereas PMAs in spleen of fish exposed to heavy metals from the Zletovska River contained predominantly lipochrome and hemosiderin pigments (Figure 2c).

Calculated values for *Vv*(PMAs, spleen) were similar among localities for fish captured in the spring season. This was not the case in the autumn season, when significant differences among localities were noted. In both rivers contaminated with metals, *Vv*(PMAs, spleen) showed increase compared to the fish from the Bregalnica River, but statistically significantly higher values for *Vv* were found only in autumn for fish from the Kriva River (Table 4). The increase of *Vv*(PMAs, spleen) in the fish from the Kriva River in the autumn season was also significant compared to the values for *Vv*(PMAs, spleen) measured in spring for the same locality. Within the age groups similar results were acquired, with increased *Vv*(PMAs, spleen) in fish spleen in autumn at the Kriva River in the group age 2-3 compared to the values for fish from the Bregalnica. There were no significant differ-

Table 4. *Relative (Vv) and total volumes (V) of pigmented macrophages (PMAs) and number of PMAs per mm² in the spleen of Vardar chub in both age groups from three differently contaminated rivers, combined for both sexes, and presented separately for each season (spring and autumn[a]*

	Vv(PMAs) %				V(PMAs) cm^3				PMAs/mm^2			
	spring		autumn		spring		autumn		spring		autumn	
Group age 2-3 years												
Bregalnica River	2.61	(1.43)	1.77	(0.87)^{p}	0.16	(0.12)	0.19	(0.08)^{p}	131.70	(111.11)	82.90	(49.85)
Zletovska River	2.96	(2.18)	3.36	(3.42)^{pq}	0.06	(0.06)	0.06	(0.10)^{p}	139.99	(91.50)	107.64	(72.12)
Kriva River	3.17	(1.94)	6.62	(1.98)^{q}	0.17	(0.11)^{P}	0.54	(0.18)^{Qq}	284.01	(168.38)*	85.57	(10.52)
Group age 4-5 years												
Bregalnica River	2.94	(1.66)	1.56	(1.30)	0.29	(0.17)	0.28	(0.52)	130.98	(92.11)	66.22	(26.97)
Zletovska River	2.31	(0.87)	n/a		0.09	(0.06)	n/a		82.84	(42.57)	n/a	
Kriva River	1.11	(0.93)^{P}	5.33	(5.18)^{Q}	0.09	(0.08)^{P}	0.50	(0.57)^{Q}	81.19	(82.42)*	75.13	(25.06)

[a]Results are presented as mean values, followed by coefficient of variation in brackets. For each value, different lowercase letters represent significant differences between rivers within the same season (read vertically), different capital letters represent significant differences between seasons within the same locality (read horizontally), and asterisk represents difference among age groups in same season and locality (read vertically) according to two-way ANOVA followed by post-hoc Newman-Keuls test.

ences among fish age 4-5 from different localities. Only seasonal differences were detected for these fish. The fish of the group age 4-5, collected in autumn from the Kriva River had higher values for *Vv*(PMAs, spleen) compared to fish from the same locality in spring (Table 4).

Values measured for *V*(PMAs) were the highest in the fish collected from the Kriva River in all groups in the autumn season and showed significantly higher values compared to the other localities in the same season, as well as compared to the values measured in spring at the same locality (Table 4).

Number of PMAs/mm^2 did not show significant differences among three rivers in either season (Table 3). The only significant difference of number of PMAs/mm^2 was observed between age groups in fish from the Kriva River. Namely, the fish age 2-3 years from the spring season had higher values for this parameter compared to the fish age 4-5 years in the same river and season (Table 4).

DISCUSSION

It is a common practice to analyze fish body masses, organ masses and organosomatic indices when assessing the general health and the condition of specific organ. In our investigation, detrimental influence of metals on fish size was obvious, judging by the lowest values of BM, TL and Fulton's CF of fish collected from the Zletovska River in both seasons (Table 2, *23,36*). The Zletovska River had high concentrations of numerous metals dissolved in the river water (Cd, Co, Cs, Cu, Li, Mn, Pb, Rb, Sr, Tl, and Zn), with very high concentrations of dissolved Cd, Zn, and Mn, especially in autumn 2012 (32). In autumn, the concentration of Zn in the water (~1.5 mg/L) was 40-110-fold lower than the LC_{50} which was reported to be 63-170 mg/L for freshwater fish *(39)*, but it exceeded the 120 µg/L criteria of US EPA for protection of aquatic organisms. Concentration of Cd (~2 µg/L) was also comparable to LC_{50} reported to be 0.8-48 µg/L for freshwater fish *(39)*. These concentrations of the mentioned metals

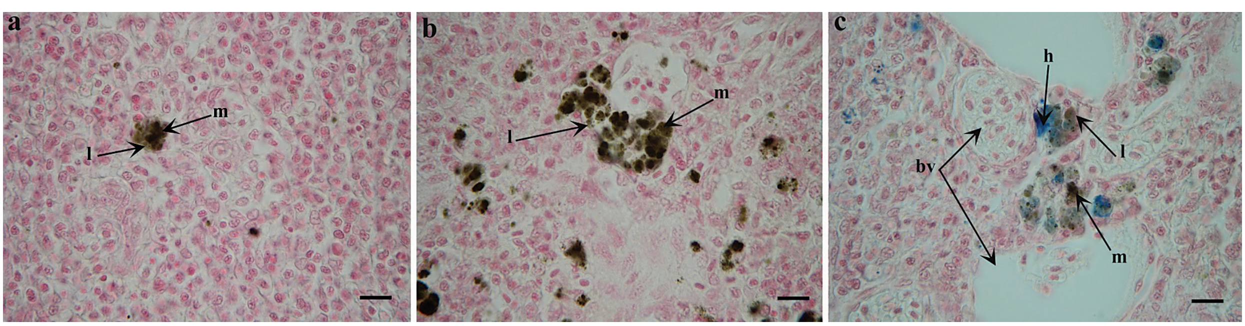

Figure 2. *Light micrograph of PMAs near blood vessels (bv), containing melanin (m), lipochrome (l) and hemosiderin (h) in spleen of Vardar chub collected in the autumn season from the Bregalnica River (a), the Kriva River (b), and the Zletovska River (c). Bar=10µm*

can disrupt growth and development of fish and interfere with many other physiological processes in metal exposed fish *(39)*. Similar inhibition of fish growth was observed in fish exposed to a mixture of sublethal concentration (50 mg/L) of Zn, Ni, Fe, Pb and Mn *(40)*. Negative correlation of fish growth was also noted with increasing concentrations of Al, Cd and Hg (41Fish collected from the Zletovska River were also shown to have higher accumulation of Cd, Cs, Pb, Rb, Sr and Tl in the liver and gills than the fish from the other two rivers *(42)*. For example, the concentrations of Cd and Pb in the water of the Zletovska River in autumn were approximately 100 times and up to 2 times higher, respectively, than in the water of the Bregalnica and Kriva rivers *(32)*. As for the Cd and Pb bioaccumulation in the liver in autumn, it was 5-25 times and 2.5-6 times higher, respectively, in the fish from the Zletovska River compared to the other two rivers; whereas in the gills it was 50-90 times and 3-21 times higher in the Zletovska River than in the Bregalnica and the Kriva rivers *(42)*. Details on the bioaccumulation of the other elements in the liver and gills of Vardar chub were published previously *(42)*. Although metals can directly inhibit fish growth, indirect influences should not be excluded. Namely, metals can have negative impact on the prey of fish as well *(43)*, thus reducing the quality of food. Also, the Zletovska River was very deficient in the nutrients compared to the Kriva River (Table 1), and nutrition deficiency might be another reason of the smaller size of fish collected at this locality. Fish growth can be limited if there is not enough protein included in the fish diet *(44)*. Therefore, the low BM, TL and Fulton's CF values for fish from the Zletovska River could be a result of combined negative influence of metal exposure and poor nutrition of fish, due to lack of organic matter (nutrients) in the river body. These differences in condition among fish from the different localities can be seen in both age groups.

In addition, the lowest values of both SM and SSI were also detected in the Zletovska River in the autumn season, which indicated that the presence of metals and nutrition deficiency had also strong negative impact on this organ and its condition for fish from group age 2-3 years. The older fish had significantly lower SSI in the spring season in the Kriva River compared to autumn. Also, spring season was the season in which the Kriva River had comparable or higher concentrations of Pb and Cd in the water than Bregalnica and Zletovska rivers (32), and increased accumulation of those metals in the liver and gills of Vardar chub from the Kriva River was noted in spring period *(32, 42)*, which confirmed that the increased concentration in the water, and consequently increased bioaccumulation of metals in fish organs has influence on fish immune system, as well. Decrease of lymphopoetic tissue caused by metal exposure in fish was previously noted *(25)* and was further confirmed in Vardar chub by the reported negative effect on the kidney, and the reduced kidney-somatic index (KSI) in the same fish specimens as studied here *(36)*. In addition to much lower concentrations of dissolved metals in the surface water of the Kriva River in the autumn season compared to the Zletovska River, the presence of nutrients in the Kriva River most likely interfered with the availability of dissolved metals at this locality. Uptake of metals in cells is dependent on the concentration that is present in the water. Namely, metals are more readily taken up by the cells if they are in free form, and not so much when they adhere to other particles and molecules *(39)*.Therefore, although both fish from the Kriva River and the Zletovska River were exposed to metals to a different degree, their accumulation in organs *(42)* and the effect on lymphopoetic tissues (spleen and kidney) corresponded to the concentrations in water and bioavailability of metals.

The microscopic analysis of Vardar chub spleen showed that the pigment content in pigmented macrophage aggregates (PMAs) varied. Hemosiderin and lipochrome were more abundant in fish exposed to metals, as opposed to fish exposed to organic matter, whose PMAs contained mostly melanin and lipochrome *(45)*. This was previously discussed in detail by Ivanova et al. *(45)*.

PMAs are part of the immune system of fish and are present in fish spleen under normal conditions, but parameters of PMAs (*Vv*, *V* and PMAs/mm^2) may undergo changes under different influences *(12, 13, 14)*. These parameters are suggested as biomarkers of pollution and its influence on PMAs and on fish health in general *(10, 13)*. Our results showed that in the autumn, the moderate presence of metals in the river water in combination with high level of nutrients has caused the significant increase of the *Vv*(PMAs, spleen) and *V*(PMAs) in the spleen of Vardar chub from the Kriva River. Similar response of *Vv*(PMAs) to pollution was observed in both age groups. It should, however, be noted that in the spring season in the fish from the Kriva River in the group aged 2-3 years, values of PMAs/mm^2 were significantly increased compared to the fish from group aged 4-5 years. This is suggesting activation of numerous small PMAs in the spring season, and their aggregation and increased volume in autumn, in the spleens of younger fish. Relative increase of PMAs/mm^2 in spleen was noted in the spring season in fish from all localities, compared to autumn. However, this increase had no statistical significance, but was in accordance with previous reports on seasonal changes of the number of PMAs/mm^2 and the volumes of PMAs in the spleen of the other fish *(7, 22, 46)*. This seasonal dynamics of values of PMAs parameters implies that the increase of PMAs number in the spleen of Vardar chub is season dependent, whereas increase of *Vv(PMAs, spleen)* or *V*(PMAs) is more influenced by pollutants exposure. Also, although relatively higher values of PMAs number was noticed in the spring season in fish from the Kriva River and in the autumn season in fish from the Zletovska River, these do not seem to contribute to volumes increase as it would be expected. Hence, inability of PMAs to aggregate due to metal exposure can be argued as was previously observed (10). This appears to be case only at high concentrations of metals because in the autumn season in the Kriva River, which has moderate metal pollution, the detected high values for *Vv*(PMAs, spleen) were accompanied by lower values for PMAs/mm^2. Increase of *Vv*(PMAs, spleen) was also noted in the spleen of fish captured in the autumn season from the Zletovska River in comparison to fish from the Bregalnica River, but it was milder. There were two possible reasons for the observed differences in the immune response of the fish from the Kriva River and the Zletovska River. One possible reason was that the increase of *Vv*(PMAs, spleen) and *V*(PMAs) in the spleen of the fish from the Kriva River occurred as an effect of the exposure to a combination of organic matter, which can trigger an immune response, and metals, which can modulate this effect. Metals are known immunomodulators, and their effect can vary from stimulating to inhibiting (18, 39). The other explanation could be that the kidney has compensated for the function of the spleen, which was reduced in size in the fish from the Zletovska River. This was indicated by the higher values of *Vv*(PMAs) in the kidney of the same Vardar chub from the Zletovska River (36). In fish, kidney and spleen are major hematopoetic organs and are considered analogues to bone marrow of other vertebrates. Although kidney is the primary site of blood cells production and spleen is the primary organ of blood cells recycling, their role is interchangeable (11). Namely, when the spleen is removed, the kidney becomes the primary site of PMAs accumulation (47).

In conclusion, the isolated effect of extremely high concentrations of dissolved metals caused the decrease of the spleen size of Vardar chub from the Zletovska River, and the reduced spleen size and/or function consequently has led to redirection of the PMAs accumulation to the kidney. Contrary, when moderate metal exposure was combined with exposure to high nutrient level in the Kriva River, more complex response was initiated, leading to significant increase of PMAs volumes in the Vardar chub spleen. Moreover, seasonal effect was also observed on PMAs volumes, with higher values of *Vv*(PMAs, spleen) and *V*(PMAs) in the spleen of Vardar chub recorded in autumn in the Kriva River, and higher PMAs number in spring. In accordance with the known fact that in the natural conditions PMAs accumulation in the fish spleen depends on the season, we have also found higher PMAs number in the spring season, and higher PMAs volumes in the autumn season, but this is dependent of the type and intensity of water contamination. Taking in consideration the possibility of immunostimulation of PMAs in one river contaminated with metals, and possibility of their imunosupression in the other, we propose that this biomarker of water pollution should be used in the monitoring as a supplement to the other routinely measured parameters, and not as an isolated biomarker of metal pollution. In addition, future studies should be made, referring to the effect of different mixtures and concentrations of metals, as well as the nutrients, on the PMAs amount and volumes.

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