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Effects of heavy metal pollution on pigmented macrophages in kidney of Vardar chub (*Squalius vardarensis* Karaman)

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RUNNING TITLE: Effect of metals on kidney macrophages

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ABSTRACT

Pollution with heavy metals may influence the immune system of fish, leading to impairment of their health or even increase their mortality. The fish kidney is one of the first fish organs to be affected by water contamination. Amounts of kidney macrophages (MACs), which are involved in fish immune response, as well as the qualitative and quantitative changes in the pigmented MACs in fish kidney, are used as biomarkers of pollution. Therefore, in this study we have evaluated relative and total volumes of trunk kidney pigmented MACs, and analyzed the pigments accumulated within them. Fish were sampled from two mining impacted rivers, Kriva and Zletovska, highly contaminated with heavy metals, and from one reference river, Bregalnica, in spring and autumn of 2012. We have observed that main pigments found in kidney MACs of Vardar chub were melanin and lipofuscin/ceroid, as well as that relative volumes of MACs ranged from 0.56-1.68%. Moreover, the results showed that relative volumes of pigmented MACs were higher in metal contaminated rivers, especially in autumn season in the Zletovska River, concurrently with extremely high metal exposure. In addition, condition factors and kidney somatic indices were found significantly lower in the Zletovska River in both seasons, autumn and spring, possibly also as a consequence of high water pollution. Our data confirm that increase in relative volumes of pigmented MACs may serve as warning sign of potential heavy metal pollution in aquatic environment.

KEW WORDS: metals pollution, macrophage aggregates, kidney, fish

INTRODUCTION

Pigmented macrophages (MACs) are the oldest known immune cells and are considered as a part of the reticulo-endothelial system and, hence, a part of the fish defense system. They are the site of centralization of foreign material and cellular debris for destruction, detoxification or reuse, the storage of exogenous and endogenous waste products, the immune response, iron storage and recycling (Agius, 1979; Agius and Roberts, 2003). According to their function they are believed to be analogues to the mammalian germinal centers (Agius, 1985; Blazer et al., 1997).

Pigmented MACs are a prominent feature in fish spleen, kidney and sometimes in liver (Agius, 1979; Blazer et al., 1994). Occurrence of MACs, their number, and degree of their pigmentation depend on the age, sex, nutrition status and health status of the fish (Agius, 1979; 1985; Agius and Roberts, 2003; Blazer et al., 1994; 1997; Jordanova et al., 2016a; Montero et al., 1999; Roberts, 1975; Schwindt et al., 2008). The liver and kidney melano MACs also appear to be related to the state of reproductive development (Elston et al., 1997; Jordanova et al., 2008; 2011).

Numerous investigators have documented increase in MACs number, size and/or pigment density in organs of fish with poor health, pathogen conditions (Agius, 1979; Elston et al., 1997; Roberts, 1975) or as a response to various environmental contaminants in field and/or laboratory studies (Agius and Roberts, 2003; Blazer et al., 1987; 1994; 1997; Broeg, 2003; Couillard and Hodson, 1996; Elston et al., 1997; Facey et al., 2005; Fournie et al., 2001; Fenoglio et al., 2005; Jordanova et al., 2016b; Schwindt et al., 2006; 2008; Wolke, 1992). Data on influence of heavy metals on MACs also exist, but according to our knowledge they are scarce (Blazer et al., 1997; Jasim, 2008; Kurtović et al., 2008; Mela et al., 2007; Rabitto et al., 2005; Reddy, 2012; Suresh, 2009; Vinodhini and Narayanan, 2009) and although they are mainly quantitative in nature, different researchers examined different portions of kidney and/or different MACs parameters, making the comparison of the results practically impossible. Although MACs are easy to identify, quantification of their size/number/volume occurrence is time consuming.

Many toxicants, including heavy metals, enter aquatic ecosystems, especially near active mines, contributing to ecosystem degradation. Taking into account that MACs are good biomarkers for evaluation of the anthropogenic impact on the environment (Blazer et al., 1997), the present study aimed to investigate pigmented

MACs in the kidney of Vardar chub (*Squalius vardarensis* Karaman) collected from two metal polluted rivers (Zletovska and Kriva; Ramani et al., 2014) and one reference river (Bregalnica). As the chub is a top predator, it is particularly vulnerable to the impact of chemical agents, namely heavy metals in our case. Specific aim of this study was to stereologically, determine the quantity of pigmented MACs in the trunk kidney of Vardar chub, through determination of their relative and total volumes. Additionally, pigments within MACs were identified, and chub condition factors (CF) and kidney somatic indices (KSI) were determined, as indicators of fish health deterioration.

MATERIAL AND METHODS

Fish sampling and dissection

Vardar chub samplings were performed in spring, in spawning season (May, June) and autumn (October) of 2012 in three rivers located in the north-eastern Macedonia: Bregalnica (n=43), as a relatively clean site if metals are concerned, contaminated mainly by agricultural runoff and municipal waste waters, and two rivers which are under direct influence of Pb/Zn mines and were proven as highly metal polluted, the Zletovska River (n=40) and the Kriva River (n=30) (Ramani et al., 2014). Detailed information on sampling sites and physico-chemical characteristics of the water of all three rivers in the time of chub samplings was previously reported by Ramani et al. (2014). Fish were collected by electro fishing (electrofisher Samus 725G) according to CEN EN 14011, 2003 standard. After capture, fish were transported from sampling sites to the laboratory in plastic container with aerated river water. In the laboratory, fish were anaesthetized with clove oil. For age determination, scales were removed just below dorsal fin. Only fish of 2^+ to 4^+ age were used for this particular study. The total length (TL) was measured, as well as body mass (BM) without gonads, to avoid the influence of the gonad development on the measured parameters. The condition factor (CF) was calculated according to the following formula: $CF = BM \times 100 / TL^3$. After biometric measurements, fish were dissected, the whole kidneys were carefully removed and their masses measured (KM). Kidney-somatic index (KSI) was calculated according to the following formula: $KSI = KM \times 100 / BM$.

Tissue collection and processing

For determination of number, as well as estimation of relative and total volumes of pigmented MACs, small pieces of the kidneys were selected from the regions bellow the dorsal fin and placed in Bouin's fixative for 48 hours. The sampled pieces were routinely dehydrated through a series of alcohols, cleared in xylene, infiltrated and embedded into paraffin. From each piece we took 5 μm thick serial sections, selecting some of them for analysis by a systematic random sampling approach, to obtain a final set of slides for analyses (about 5 per fish). Sections for quantitative analyses were stained with Perl's method.

Macrophage quantification

From each selected section, 15-18 systematically sampled fields were observed and MACs were quantified at a final magnification of 400×; the first field was randomly selected. On average, 100 fields per kidney (i.e. per fish) were selected and studied. A classical unbiased stereological technique based on manual point counting (Freere and Weibel, 1967) was used to estimate the relative volume of pigmented MACs within the organ (expressed as percentage), according to the following formula:

$$V_V\ (structure,\ reference) = V_V\ (s,\ r) = [P(s) \times 100] \div P(r),$$

in which V_V (s, r) is the percentage of the total volume of a reference space occupied by one particular given structure within that space, P(s) is the total number of test points lying over the reference space (in this study complete kidney tissue), and P(r) is the total number of points falling over a particular structural component. Point counting was directly made on a light microscope, and for that a square lattice glass grid with 180 points was inserted into the left ocular.

For the estimation of total volume of the pigmented MACs the following formula was used:

$$V(s) = V_V\ (s, r) \times V(r)$$

Statistical analyses

The data are presented as group means obtained from individual fish values, and accompanied by the respective coefficients of variation (CV = SD / Mean). For statistical analyses, the software STATISTICA 7.0 for Windows was used. After checking the normality and homogeneity of variances of the data sets, data were analyzed by two-way ANOVA/MANOVA. Whenever the ANOVA disclosed significant results, comparisons among the rivers and seasons were performed using the post-hoc Tukey's test. Differences were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Condition factors and organo-somatic indices are standard parameters in fish studies, which indicate health status and well-being of individuals. In our study, CFs, or relationship between mass and length, and KSI, expressed as percentage size of kidneys, reflected the status of chub organisms or their kidneys, respectively, in mining impacted rivers. According to Farkas et al. (2001), measurement of CF is especially important when comparison between groups of individuals collected in different seasons and years is made. Our results have indicated that all three parameters, BM, CF and KSI, showed significantly lower values in highly metal contaminated Zletovska River compared to reference Bregalnica River, as well as compared to the other, less metal contaminated, mining impacted Kriva River (Table 1).

Numerous field and laboratory studies have been concentrated on the effects of anthropogenic and natural-occurring chemicals in the environment on the health of aquatic organisms. Some of them indicated changes in CFs and organo-somatic indices, or both, as a reflection of exposure to different pollutants and contaminants in the water (van der Oost et al., 2003). The condition of the whole body, and of the organs, as determined by CFs and organo-somatic indices, respectively, is sometimes indicative of toxicant effects (Myers et al., 1992) and can provide information on potential pollution impact in ecosystem. Accordingly, as we have previously reported for CFs, low values of both CFs and KSI observed in the Zletovska River could be associated with extremely high metal exposure in the water of that river in the time of chub sampling, as well as with probable insufficient fish nutrition (Barišić et al., 2015; Jordanova et al., 2016c). Investigations on KSI are scarce compared with the other organo-somatic indices because the removal of the kidney is a difficult procedure (Pulsford et al., 1995). Still, some information on pollutant effect on KSI is available. For example, long-term exposure to arsenic affects KSI (Ghost et al., 2007) and reduces head kidney somatic index (Datta et al., 2009). Therefore, taking in consideration high water contamination with metals in the Zletovska River, we can presume that the decrease of CFs and KSI that we have observed was very likely the result of the negative influence of heavy metals on chub health and condition.

Furthermore, histological analysis has shown that in chub kidney pigmented MACs were randomly distributed throughout the hematopoietic tissue (Fig. 1). By use of Perl's method, differential pigment composition was visualized within MACs, based on the fact that applied stain dyes melanin, the melanosome pigment derived from tyrosine metabolism, into brown-black color. On the other hand, hemosiderin, as protein-bound iron pigment, is colored blue, whereas lipogenic pigments derived from oxidation of unsaturated lipids ceroid/lipofuscin are yellow-tan (Agius and Roberts, 2003). In our case, pigmented MACs mainly varied in color from dark brown to yellow, which indicated that main pigments located within them were melanin and lipofuscin/ceroid, whereas hemosiderin could be found extremely rarely. Relatively few studies on the pigment composition of fish kidney MACs have been reported, and their results varied depending on the fish species. For example, in the kidney of *Salmo letnica* only pigment melanin could be found in pigmented MACs (Jordanova et al., 2011). The microscopic evaluation of the kidney of the *Dicentrarchus labrax* also showed that in the most fish dark brown to black pigments were present within MACs (Kurtović et al., 2008). Some fishes, for example *Tilapia mossambica,* normally lack lipofuscin (Suresh, 2009), although lipofuscin generally appears to be the most common pigment in fish MACs (Agius, 1985; Agius and Roberts, 2003). Moreover, exposure to sublethal concentrations of Cd, Pb, Cr and Ni in solution resulted in the occurrence of MACs with lipofuscin accumulation in kidney of *Cyprinus carpio* (Vinodhini and Narayanan, 2009).

Concerning the MACs quantity, the examined specimens of Vardar chub generally contained low amounts of MACs. We have estimated stereologically relative and total amounts of pigmented MACs and according to these evaluations, 0.56-1.68% of kidney tissue was occupied by pigmented MACs (Table 2). For comparison, in *Salmo letnica*, where the same methodology was applied, much higher percentage of trunk kidney tissue was occupied with MACs, up to 7.48% (Jordanova et al., 2011).

If the locality was considered, generally higher relative volumes were noted in the kidneys of fish collected from the rivers contaminated with heavy metals compared to fish from the reference site (Fig. 1), but the difference was statistically significant only between the Zletovska River and Bregalnica River in autumn season (Table 2). Lower exposure to metals observed in the mining impacted Kriva River (Ramani et al., 2014) did not provoke MACs response of the same intensity as in the Zletovska River. This finding was in accordance with concentrations of dissolved heavy metals in the river-water, which were measured in the same time when chub were collected. Namely, the highest concentrations of heavy metals were also noted in the Zletovska River, being higher in autumn than in spring (Ramani et al., 2014). Similarly to kidney MACs response, higher frequency and intensity of histopathological alterations was also noted in the gills and liver of the same chub from the Zletovska River compared to chub from the other two polluted rivers (Barišić et al., 2015; Jordanova et al., 2016c).

However, the observed differences in MACs relative volume do not have to be the consequence only of the changes in metal exposure, but could also have a physiological background. Mizuno et al. (2002) suggested that differences in seasons could influence MACs deposition in trunk kidney. For example, Saha et al. (2002) reported that the immune systems are more effective during spawning season. Therefore, MACs response obviously depends on seasonal changes in fish physiological status, and thus it is better to assess metal pollution effect on MACs response in chub kidney during the post-spawning period, when the influence of fish physiological status is less pronounced. This could also be the explanation why the differences between sites in MACs relative volume were more evident and significant in autumn than in the spring, spawning period. Contrary to relative volumes, significant differences between sampling sites were not noted for total volumes of pigmented MACs in trunk kidney.

Previous studies have also shown that higher number and/or size of phagocytic areas of MACs occur in the kidney of fish experimentally exposed to heavy metals compared with control fish (Jasim, 2008; Kurtović et al., 2008; Mela et al., 2007; Rabitto et al., 2005; Reddy, 2012; Suresh, 2009). Moreover, MACs response depends on the type of metals. For example, in the spleen number of MACs in fish treated with food rich with Zn and Fe decrease, but increase if fish were treated with copper rich food (Manera et al., 2000). On the other hand, chronic exposure to arsenic led to reduction in melano-macrophage population in head kidney, but increased hemosiderin accumulation (Datta et al., 2009). Contrary, exposure to Pb(II) resulted in the increase of the melano-macrophage centres (Rabitto et al., 2005). Exposure to

Cd has also influenced MACs number, which varied depending on doses and durations of the treatments (Jasim, 2008). Although, according to currently published information, some metals tend to increase and the others tend to decrease MACs pool, our results, which were gathered from wild population of Vardar chub inhabiting river contaminated with mixture of metals, indicated that in the kidney of this particular fish species simultaneous exposure to high concentrations of large number of heavy metals undoubtedly causes increase of relative volumes of pigmented MACs, making them good biomarkers of metal pollution.

CONCLUSIONS

Pollution is worldwide problem. Many toxicants, including heavy metals, enter aquatic ecosystems, contributing to their degradation, as well as to promotion of toxic effects on aquatic organisms, such as fish. Based on our results, qualitative and quantitative changes in pigmented MACs in fish kidney can be used as histological biomarkers to assess degradation of fish health, specifically of Vardar chub, due to exposure to metal pollutants. Relative volumes of pigmented MACs in kidneys of Vardar chub could be applied as useful biomarker of metal pollution due to their increase after chub exposure to high metal concentrations. Since observations on pigmented MACs amount in relation to exposure to heavy metals in natural environment, as well as in the laboratory conditions, are still quite limited, it would be useful in the future to examine dose dependent effects of mixtures of various metals, as well as of single metals on MACs response. Moreover, as different researchers investigate different MACs parameters, standardization of the protocols is of prime importance.

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Conflict of interest: NONE

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Tables

Table 1. Body masses (BM), condition factors (CF) and kidney-somatic indices (KSI) of Vardar chub (*Squalius vardarensis*) sampled in spring and autumn of 2012 from reference Bregalnica River and two rivers impacted by heavy metal pollution, Zletovska and Kriva.

	BM (g)		CF		KSI %	
Location	Spring	Autumn	Spring	Autumn	Spring	Autumn
Bregalnica	69.02 (0.61) ^{a}	68.82 (0.38) ^{a}	1.14 (0.09) ^{a}	1.06 (0.06) ^{a}	0.76 (0.29) ^{a}	0.82 (0.24) ^{a}
Zletovska	28.67 (0.58) ^{b}	36.04 (0.97) ^{b}	0.99 (0.07) ^{b}	0.87 (0.08) ^{b}	0.55 (0.26) ^{b}	0.46 (0.16) ^{b}
Kriva	56.44 (0.48) ^{a}	46.43 (0.28) ^{ab}	1.13 (0.07) ^{a}	0.97 (0.05) ^{ab}	0.87 (0.16) ^{a}	0.80 (0.12) ^{a}

Data are expressed as means, with coefficient of variations in brackets. Within each column, values with different superscript letters are significantly different ($p<0.05$), according to the post-hoc Tukey's test.

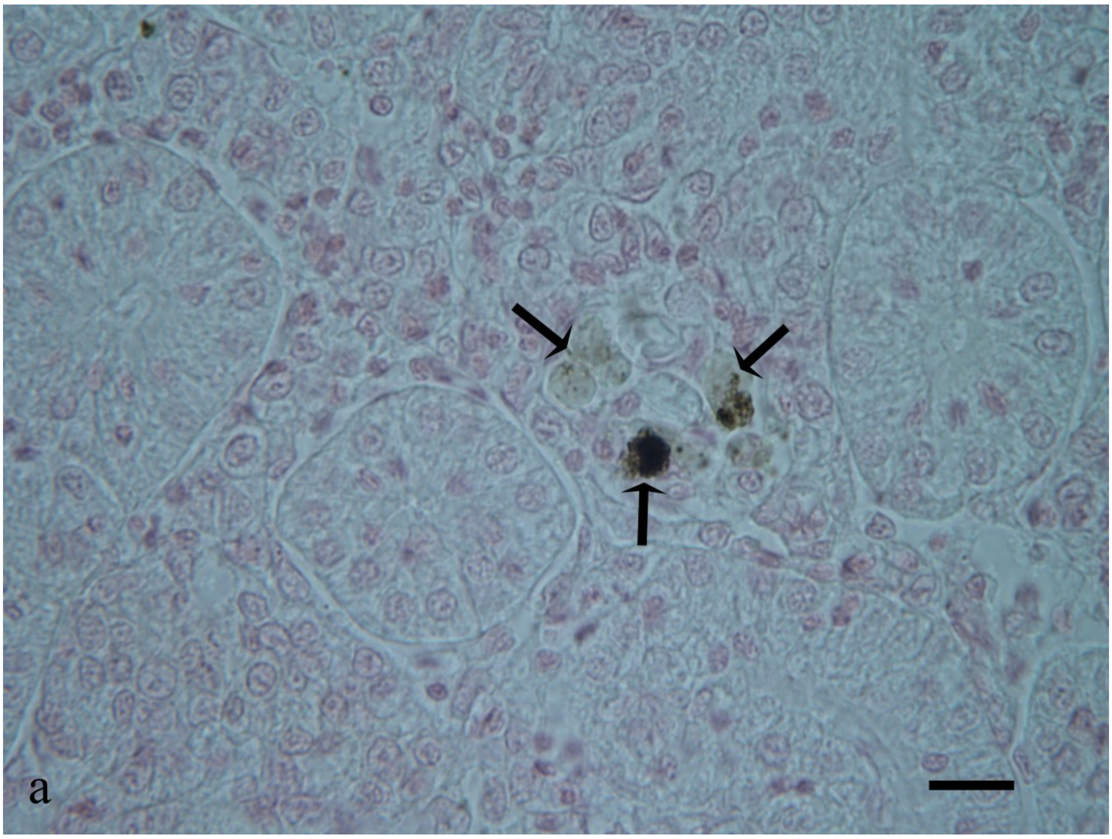
Table 2. Relative volumes (Vv) and total volumes (V) of pigmented macrophages (MACs) in the kidney of Vardar chub (*Squalius vardarensis*) sampled in spring and autumn of 2012 from reference Bregalnica River and two rivers impacted by heavy metal pollution, Zletovska and Kriva.

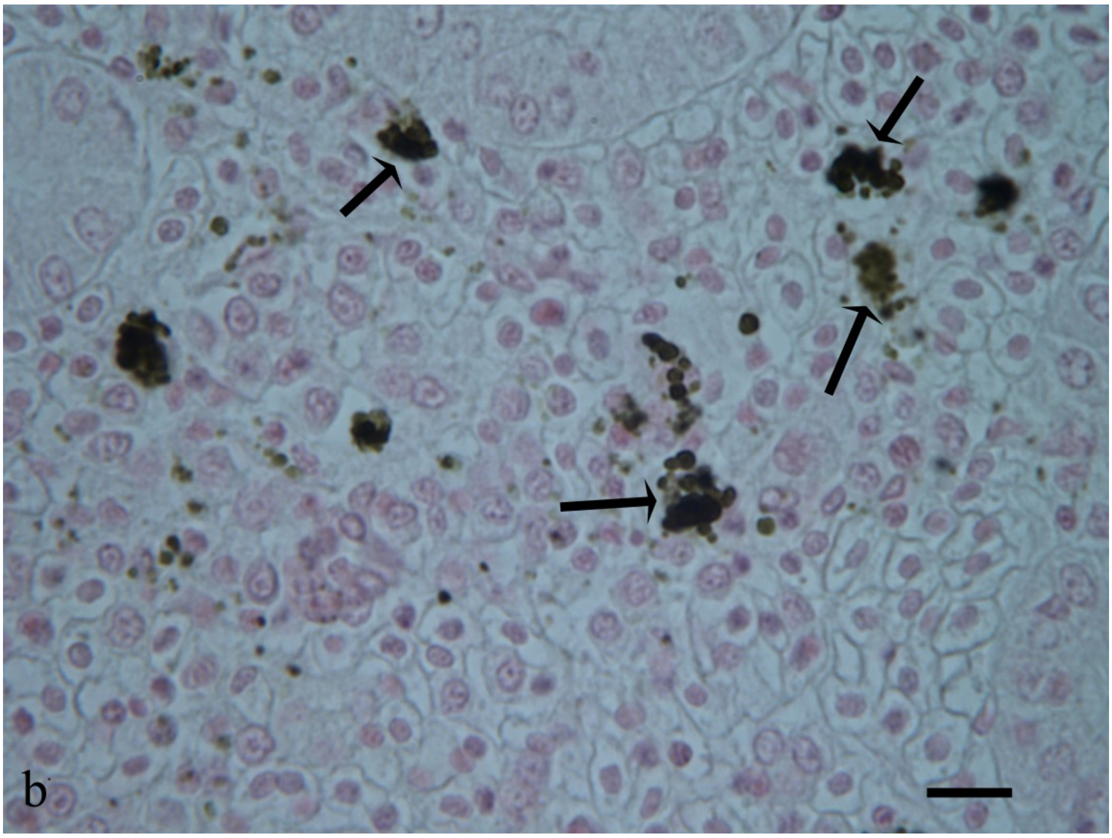
	V_v(MACs) %		V (MACs) cm^3	
Location	Spring	Autumn	Spring	Autumn
Bregalnica	0.80 (0.86)	0.56 (0.81) ^{a}	0.53 (1.27)	0.32 (0.89)
Zletovska	1.11 (0.75)	1.68 (0.46) ^{b}	0.21 (1.37)	0.28 (1.03)
Kriva	0.92 (0.83)	0.86 (0.74) ^{ab}	0.47 (1.16)	0.28 (0.55)

Data are expressed as means, with coefficient of variations in brackets. Within each column, values with different superscript letters are significantly different ($p<0.05$), according to the post-hoc Tukey's test.

Figure legend

Figure 1. Light micrographs of Perl’s stained sections from the kidney of Vardar chub, showing the evident differences between (a) fish in the reference site with fewer pigmented macrophage (arrows), and (b) fish from Zletovska River with much extensive macrophage accumulations. Bar line = 10µm.

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