The effect of different pollutants exposure on the pigment content of pigmented macrophage aggregates in the spleen of Vardar chub (Squalius vardarensis Karaman, 1928)

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The effect of different pollutants exposure on the pigment content of pigmented macrophage aggregates in the spleen of Vardar chub (*Squalius vardarensis* Karaman, 1928)

Running title: Pigment content of the pigmented macrophage aggregates in the chub spleen

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Abstract

Pigmented macrophage aggregates (MAs) are known to change under influence of various factors, such as aging, season, starvation and/or pollution. In this study, changes in the pigment content of the MAs in the spleen of Vardar chub (*Squalius vardarensis*, Karaman) (n=129) collected in spring and autumn, from three rivers with different pollution impact was examined: Zletovska River (metals), Kriva River (metals and municipal wastewater), Bregalnica River (municipal wastewater). Collected data revealed increased relative volume and number of MAs containing hemosiderin under the influence of metals, significant in autumn ($p<0.05$). In chub exposed to metals combined with municipal wastewater, significant increase of lipochrome accumulation in MAs in autumn, melanin in MAs in fish captured in the spring season, and number of splenic MAs containing combination of melanin and lipochrome was noted. Volumes and number of MAs containing combination of hemosiderin and lipochrome increased in spleen of fish captured in autumn from both Zletovska River and Kriva River, most likely due to the contribution of hemosiderin and lipochrome, respectively. Values measured for the various pigments in splenic MAs in fish captured from Bregalnica River, were overall closer to the values measured for fish captured from Kriva River. Notably, melanin and lipochrome are more likely to be found in fish from waters influenced by municipal wastewater (organic pollution) and haemosiderin in fish spleen from water influenced by mining activity (heavy metals pollution).

Key words: lipochrome, melanin, hemosiderin, macrophage aggregates, heavy metals

Highlights:

- Impact of heavy metals on pigment accumulation in MAs in spleens of Vardar chub was assessed.
- Season dependent changes in melanin accumulation was observed.
- Heavy metals exposure results in increased hemosiderin accumulation.

Introduction

Rivers in R. North Macedonia are under increased pressure of pollution, and nearly all rivers in the region are pollution impacted in various degrees, especially in the north-eastern region where heavy metal input is present from local mining activity (Ramani et al., 2014). The negative effects of heavy metals were already noted (Zelikoff, 1993; Vinodhini and Narayanan, 2009; Kennedy, 2011; Tchounwou, Yedjou, Patlolla and Sutton, 2012), and therefore monitoring is advised to avoid and prevent ecosystem degradation. Among aquatic organisms, fish are often used as bioindicators, because as the main predators they can bioacumulate and bioconcentrate contaminants, such as metals, and reflect the condition on several trophic levels (van der Oost, Beyer and Vermeulen, 2003; Oliveira Ribeiro, Vollaire, Sanchez-Chardi and Roche, 2005; Maceda-Vaiga, Monroy and de Sostoa, 2012). Changes in the fish health caused by pollution are most often noticed on organ and/or organism levels, which is unfavorable (James, Sampath, Jothilakshmi, Vasudhevan and Thangarathinam, 2008; Price, 2013). The cells of the immune system are a good candidate for the purpose of early warning signals, as they are one of the first cells that respond to changes in the environment (Gil and Pla, 2001; Skouras, 2002). Spleen is part of the immune system of fish, and functions as a filter of the blood, which is trapping and processing antigens. This function is performed by specialized cells, macrophages, which after phagocytizing the debris, migrate to form aggregates (Zapata, Chiba and Varas, 1996; Fournie, Summers, Courtney and Engle, 2001). As a product of processing the debris and the other particles, macrophages accumulate various pigments (lipochrome, melanin, and hemosiderin) in their cytoplasm, which is why they are called pigmented macrophage aggregates (Wolke, George and Blazer, 1985; Wolke, Murchelano, Dickstein and George, 1985; Wolke, 1992; Agius and Roberts, 2003). Although many organs, such as liver and kidney, can accumulate macrophage aggregates, spleen is considered to accumulate them more readily (Kurtović, Taskeredžić and Taskeredžić, 2008; Russo, Yanong and Terrell, 2007). Also, the organ of MAs accumulation depends on the fish species. Namely, some fish might not have MAs in liver or kidney, and in most species, spleen is the main organ of accumulation of MAs (Agius, 1979; Blazer, Wolke, Brown and Powell, 1987). The formation of different pigments has specific biochemical pathways, so it was suggested that the pigment contents and/or their ratios can vary depending on the exposure to different contaminants and can therefore be used as biomarkers (Wolke, George and Blazer, 1985). Lipochrome is a result of oxidative polymerization of unsaturated fatty acids and proteins, and it can accumulate in the cells normally with ageing, but is also associated with excessive tissue destruction (Terman and Brunk, 1998; Terman, Gustafsson and Brunk, 2007; Yin, 1996). Lipochrome appears as waxy-yellow pigment in the spleen sections (Wolke, George and Blazer, 1985; Wolke et al., 1985; Wolke, 1992; Agius and Roberts, 2003). Melanin is dark brown to black pigment; it is an endogenous polimer which results from quinonic precursors and has the ability to neutralize free radicals, cations and other potential toxins (Wolke, George and Blazer, 1985, Wolke et al., 1985; Zuasti, Jara, Ferrer and Solano, 1989; Wolke, 1992; Agius and Roberts, 2003). Melanin can also act as bactericide (Wolke, George and Blazer, 1985; Wolke et al., 1985; Zuasti et al., 1989; Wolke, 1992; Agius and Roberts, 2003). Hemosiderin is a form of a protein for iron storage (Agius, 1979), and it is increased when the tissue is saturated with ferritin (Agius and Roberts, 2003). Because of its association to Fe^{3+}, hemosiderin appears blue in the spleen sections stained with Perls' stain (Wolke, George and Blazer, 1985; Wolke et al., 1985; Wolke, 1992; Agius and Roberts, 2003).

Although the changes in the size and area parameters of MAs, as well as in their number under different conditions and in various organs were already noted (Wolke, George and Blazer, 1985; Meinelt, Krüger, Pietrock, Osten and Steinberg, 1997; Manera, Serra, Isani and Carpené, 2000; Giari, Manera, Simoni and Dezfuli, 2007; Mela et al., 2007; Suresh, 2009; Rebok, Jordanova and Tavciovska-Vasileva, 2011; Reddy, 2012; Barstet al. 2015; Jordanova et al. 2016; Jordanova et al., 2017), relatively few of the published studies has addressed the changes in the pigment content (Agius, 1981; Wolke, George and Blazer, 1985; Blazer, Wolke, Brown and Powell, 1987; Haaparanta, Valtonen, Hoffmann and Holmes, 1996; Patey, Couillard, Pierron, Beaudrimont and Couture, 2017). This is especially true when investigation is conducted under the field conditions. Moreover, in a study of changes in macrophage aggregates in different organs of perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) as an indicators of pollution, inconclusive results in correlation to the exposure were reported (Haaparanta et al. 1996) in contrast to the observed change in hemosiderin accumulation in eel from contaminated sites and the difference in sensitivity in different species (Patey et al. 2017). Moreover, different patterns of pigment accumulation were seen in spleens of shorthorn sculpins (Dang et al. 2019). Namely, hemosiderin and lipofuscin tended to be homogenously distributed in MAs, while melanin containing macrophages formed clumps (Dang et al. 2019). In other study of the histopathology of the livers of *Gymnotus inaequilabiatus* in relation to parasite infection, hemosiderin was the most abundant pigment in MAs of in this organ, whereas distribution of lipochrome and melanin pigments was mild and variable (Dezfuli, Manera, DePasquale, Pironi and Giari, 2017).

Therefore, the goal of this investigation was to determine if the pigment content in splenic MAs is changing when chub are exposed to various contaminants in natural conditions, where various interactions between environmental factors can occur.

Materials and methods

Study area and fish sampling

In this study, the pigment content of MAs from spleens of Vardar chub (n=129) captured from three different localities, in two different seasons (spring and autumn 2012) was examined. The collection of fish was a part of a project, therefore, detailed description of water chemistry at three localities at the time of collection was previously reported by Ramani et al. (2014), whereas detailed description of the fish collection and dissection, as well as determining the age, the morphometrics and condition of fish and gills, liver, gonads and kidney was reported by Barišić et al. (2015), Jordanova et al. (2016, 2017) and Dragun et al. (2019). The collected data is presented here separately for both seasons and sexes (Table 1), and was used for supporting the interpretation and conclusions arisen from this work.

The first collection point was at the Zletovska River, which is burdened with heavy metals (Ramani et al., 2014). In the water collected in the spring season high values for Cd (0,272 μgL^{-1}), Zn (197,0 μgL^{-1}) and Mn (351,9 μgL^{-1}) were measured (Ramani et al., 2014), and for the autumn season these values were manyfold higher (Cd 2,00 μgL^{-1}, Zn 1507 μgL^{-1}, Mn 2527 μgL^{-1}). In autumn, Pb concentration (0,748 μgL^{-1}) was also increased compared to spring (0,313 μgL^{-1}), but these values were within the proposed environmental quality standards (Ramani et al., 2014). The second collection point was at the Kriva River, which has presence of both heavy metals and household wastewaters (Ramani et al. 2014, Kapetanović et al. 2014). In this river only Cd concentrations in spring were above recommended values (spring: Cd 0,270 μgL^{-1}, Zn 22,07 μgL^{-1}, Mn 9,90 μgL^{-1}, Pb 1,95 μgL^{-1} autumn: Cd 0,03 μgL^{-1}, Zn 3,81 μgL^{-1}, Mn 9,65 μgL^{-1}, Pb 0,42 μgL^{-1}). Highest total coliforms count was detected in the Kriva River in autumn (435170 MPN/100mL), compared to the Bregalnica River (15214 MPN/100mL) and the Zletovska River (3864 MPN/100mL) (Ramani et al. 2014, Kapetanović et al. 2014). The third point of collection was at the Bregalnica River and characterized by fecal and organic contamination (Spasovski, 2011; Ramani et al., 2014; Stipaničev et al., 2017). Metal concentrations in both seasons in the Bregalnica River were below recommended values (spring: Cd 0,032 μgL^{-1}, Zn 4,97 μgL^{-1}, Mn 13,27 μgL^{-1}, Pb 0,69 μgL^{-1} autumn: Cd 0,02 μgL^{-1}, Zn 6,14 μgL^{-1}, Mn 9,65 μgL^{-1}, Pb 0,42 μgL^{-1}) (Ramani et al. 2014). Bioaccumulation of heavy metals in liver and gills was obvious, especially at the Zletovska River (Cd, Pb, Cs, Tl, Rb,)in both seasons (Dragun et al., 2019). At the Kriva River, higher bioaccumulation of Cd and Pb in livers and gills was observed only in the spring, which was consistent with water contamination only in that period (Dragun et al., 2019). In the Bregalnica River, increased bioaccumulation of V was observed, consistent with water contamination with agricultural runoff (Dragun et al., 2019). Fish were collected by the method of electro-fishing, and were then transported to the laboratory in oxygenated tanks.

Tissue collection and processing

Following the euthanization (Clove oil, Sigma), fish total length (in cm) and weight (g) were measured, and values for Foulton's CF were calculated. Spleens were removed, measured and processed for paraffin sections. Spleen weight was used in determining the splenosomatic index for each fish. Paraffin sections 5μm from each fish, were cut on manual rotary microtome. Five sections were chosen for analysis by systematic random sampling (SRS) method. Several stains were used for confirmation of the pigments on sections from eight chub spleen: Masson-Fonatana, Schmorl's and melanin bleaching confirmed the presence of melanin pigment, whereas Sudan black and Zeihl Neelsen were used for confirmation of lipochrome pigments. Perls' stain was chosen as the best method that allows the visualization of the three pigments (melanin; lipochrome; and hemosiderin) in MAs simultaniously.

Stereology measurements

The relative volume (Vv), total volume (V) and number of MAs per mm^2 (MAs/mm^2) containing the various combination of the pigments in the spleen of fish were measured manually, with classical stereological method based on point counts (Freere and Weibel, 1967). Sections were analyzed by light microscopy, at magnification of 400×, using an ocular with a grid of 180 points. Each point from the grid that overlapped the pigmented macrophages was counted. Due to limited resources of our laboratory, it was not suitable for separate and isolated measurement of each pigment we differentiated the MAs by the combinations of the pigments they contained. Namely, we separately counted points that overlap MAs that contained only melanin; only lipochrome; or just hemosiderin, as well as their combinations: melanin+lipochrome; melanin+hemosiderin; lipochrome+hemosiderin; melanin+lipochrome+hemosiderin. The types of pigments contained in the MAs were determined visually, according to their characteristic colorization with Perls' method. These differential counts were conducted on at least 50 fields per fish and were chosen by SRS from 5 sections. The counts were used for calculating the relative volume of the MAs containing different pigments and their combinations, by the following formula:

$$Vv\ (\%) = [P(s) \times 100] / P(r),$$

P(s) is the number of points overlapping with MAs, noted separately for each pigment combinations, P(r) is the number of points overlapping with the parenchyma of the spleen. In addition total volume estimates were included, as it provides a global estimate that is independent of organ volume changes (Matsche, Blazer and Mazik, 2019), and was calculated by multiplying the relative volume counts with the organ volume V(spleen):

$$V\ (cm^3) = Vv \times V(spleen),$$

The same grid with estimated area of 21,025 $\times 10^{-3}$ mm^2 was used for determination of the number of MAs/mm^2 containing each pigment separately or their combinations, using the following formula:

$$MAs/mm^2 = No.of\ MAs/(AF \times 21{,}025 \times 10^{-3})$$

No. of MAs is the total count of MAs with different pigment contents in the grid frame (not counting the overlaps on the left and down corner), and AF is the number of analyzed fields per individual fish.

Statistical analysis

Statistical analysis was conducted with Statistica 8.0 for Windows (Stat Soft) on $\log_{10}$-transformed data, using two-way ANOVA to test the effect of season, sex, and locality, followed by post hoc Tukey test. Results were considered significant at $p < 0.05$. Redundancy analysis (RDA) in the software XLSTAT 2014 was conducted for reducing multidimensionality and to make connections between pollutants and MAs observations. Data for metals concentrations at point and time of collection of fish, previously reported by Ramani et al. (2014), and for fish condition, previously reported by Jordanova et al. (2016), was used in the RDA analysis. MAs scores and fish condition were used as response variables, and water pollutants, locality and season as explanatory variables.

Results

Visual inspection of slides showed that MAs in spleen of fish collected in both seasons from the Bregalnica River contained mostly lipochrome and melanin pigments, with more compact aggregates seen in fish spleen in autumn than in the spring season (Figure 1a, b). In the spring season, small MAs containing predominantly melanin was noted in the spleen of the fish captured from the Kriva River

(Figure 1c). In the fish from the Zletovska River, captured in the spring, melanin was often found in combination with both hemosiderin and lipochrome (Figure 1e). Microscopic analysis of spleen of the fish collected from the Zletovska River and the Kriva River in autumn revealed that MAs were hemosiderin laden, accompanied with the accumulation of lipochrome and melanin in the fish from the Kriva River (Figure 1d, f).

The results for the number and volume measurements for MAs with different pigment combinations and the significant difference among groups are presented in Table 2. Graphical representation of the relative amount of MAs with each pigment combination was also presented for juxtaposition (Figure 2). Parameters of MAs (Vv, V and MAs/mm^2) laden with different combinations of pigments indicated that pigment content of MAs varied in different seasons, and was also dependent on the pollution exposure. Namely, values of Vv, V, and MAs/mm^2 for MAs containing exclusively lipochrome were significantly higher for the fish from the Kriva River collected in autumn, compared to the values measured for the fish from the same locality in the spring season (Table 2, Figure 2). Also, this increase of the values of MAs containing lipochrome measured in autumn for the fish from the Kriva River was accompanied with significantly higher values of Vv and V of MAs containing hemosiderin+lipochrome and of MAs containing hemosiderin+melanin+lipochrome, as well as the MAs/mm^2 for MAs containing hemosiderin+lipochrome (Table 2, Figure 2). Vv and V of MAs containing hemosiderin, and Vv of MAs containing hemosiderin+lipochrome also showed significantly higher values in autumn season compared to the spring season, in the fish from the Zletovska River (Table 2, Figure 2). On the other hand, values for melanin laden MAs were higher in the fish caught in the spring season, especially in the fish captured from the Kriva River. In those fish values of all parameters for MAs containing melanin, as well as of MAs/mm^2 for MAs containing melanin+lipochrome were significantly higher in the spring, compared to autumn (Table 2, Figure 2). In the fish from the Bregalnica River in the spring season, Vv of MAs containing hemosiderin+lipochrome and MAs/mm^2 for MAs containing hemosiderin+melanin+lipochromewere higher compared to autumn (Table 2, Figure 2). It should be noted, that although each pigment was not measured separately, the visual inspection showed that contribution of lipochrome and melanin in analyzed combinations surpassed the contribution of hemosiderin in the spleens of the fish collected in the Bregalnica River (Figure 1a, b), which was not the case in MAs observed in the fish from the Zletovska River, that contained predominantly hemosiderin (Figure 1e, f).

Differences among localities with different pollution impact were also observed. In autumn, in the fish from the Kriva River and the Zletovska River, which were heavily metal polluted, increased accumulation of hemosiderin was observed. Values of Vv and V of MAs containing hemosiderin+lipochrome and MAs containing hemosiderin+melanin+lipochrome detected in the fish from the Kriva River, Vv and MAs/mm^2 of MAs containing hemosiderin+lipochrome, as well as Vv, V, and MAs/mm^2 of MAs containing hemosiderin detected in the fish from the Zletovska River were significantly higher compared to the values of the corresponding parameters measured in the fish from the Bregalnica River. Values of Vv and V of MAs containing hemosiderin were also significantly higher in the fish from the Zletovska River compared to those measured in the fish from the Kriva River (Table 2, Figure 2). On the other hand fish captured from the Kriva River in autumn, which was characterized by milder heavy metal pollution (Ramani et al., 2014), had higher values of Vv and V of MAs containing lipochrome compared to those measured for the fish from the Zletovska River. In the spring season, the highest values of Vv and MAs/mm^2 for MAs containing lipochrome were measured in the fish from the Bregalnica River compared for values measured in fish spleen from the Kriva River, and were accompanied by significantly higher values, for all parameters for MAs containing hemosiderin+lipochrome. Similarly, Vv of MAs containing hemosiderin+lipochrome and of MAs containing hemosiderin+melanin+lipochrome, as well as MAs/mm^2 of MAs containing hemosiderin+lipochrome in the spleen of the fish from the Zletovska River were significantly increased compared to the same parameters in the fish captured from the KrivaRiver. This was not the case with V of MAs containing hemosiderin+lipochrome, which was significantly higher only in the fish from the Bregalnica River compared to both the fish from the Zletovska and from the Kriva River. Melanin appeared to be more prominent in MAs in the fish from the Kriva River, which was reflected in the significantly higher values for Vv, V, and MAs/mm^2 of MAs containing melanin, as well as Vv and MAs/mm^2 of MAs containing melanin+lipochrome (Table 2).

From the RDA analysis, the following information was extracted. The first two factors accounted for 74,80% of the total variance. Factor 1 accounted for 43,26% of the variation, and was associated with increased parameters of lipochrome, melanin and melanin+lipochrome laden MAs. These were influenced by presence of Pb in the spring period in the Kriva River (Table3; Figure 3). Parameters for hemosiderin laden MAs were distributed along the F2 axis and accounted for 31,54% of the variation. Also, negative correlation between SSI, Foulton's CF and volumes and number of hemosiderin laden MAs are explained by Cd, Zn, and Mn present in the Zletovska River. The fish from the Bregalnica River in autumn are associated with lipochrome laden MAs accumulation and bigger spleens (Table 3; Figure 3).

Discussion

The difference in the activity of MAs in different seasons was emphasized through the differences of the pigment accumulation within MAs. Although all pigments were present in the fish from all three localities and in both seasons, melanin was more prominent in the fish from all three rivers caught in the spring season and thus seems to be seasonally dependent. Melanin is a complex polymer and has a protective role in neutralizing free radicals in situations when the enzyme-based system is less active (Wolke, George and Blazer, 1985; Agius and Roberts, 2003), and appears in MAs of fish exposed to lower temperatures (Agius and Agbede, 1984; Wolke, George and Blazer, 1985). Therefore, seasonal differences in melanin accumulation are mostly attributed to differences in temperatures, starvation during the winter period and/or breeding dependent changes in metabolism (Manera et al. 2000; Mizuno, Misaka, Miyakoshi, Takeuchi and Kasahara, 2002; Jordanova, Rocha, Rebok and Rocha, 2011; Rebok, Tavčiovska-Vasileva and Jordanova, 2015). In this vain, it should be considered that spring sampling follows after the long period of low winter temperatures and lower metabolic rates, whereas the autumn sampling follows after several warm summer months with higher feeding and metabolic rates. Also, the spring increase of melanin-laden MAs was the highest and significant in the fish from the Kriva River, which were exposed to combination of organic contaminants and high concentrations of Cd (0.270 μgL^{-1}) and Pb (1.85 $\mu g\ L^{-1}$) in the water (Ramani et al. 2014), but accumulation of these metals in livers and gills of chub was also evident (Dragun et al., 2019). Metals in the water are one of the oxidative stress inducing agents (Sevcikova, Modra, Slaninova and Svobodova, 2011). Given the proposed function of melanin as a neutralizer of free radicals at low temperatures, its increase in the fish exposed to metals could be expected, especially considering that melanin accumulation in fish exposed to Cd was already noted from other authors (Jasim, 2008; Reddy, 2012).For example, Cd disrupts the electron transport chain in mitochondria, and creates superoxide radicals; it also displaces Cu and Fe, and interferes with the function of antioxidant enzymes (Sevcikova et al., 2011).This can explain the lipochrome and melanin accumulation. According to the RDA, Pb seems to be explanatory factor for melanin accumulation in the fish from the Kriva River in spring (Figure 3). However, such prominent increase of melanin within MAs was not observed in the fish from the heavily metal contaminated Zletovska River, but rather the accumulation of hemosiderin laden MAs is associated with this locality. The increased accumulation of hemosiderin and lower values for SSI and Foulton's CF in the fish from the Zletovska River was indicative the negative impact of the high concentrations of several metals, e.g. Cd (0.272 μgL^{-1}), Mn (351.9 μgL^{-1}) and Zn (197.0μgL^{-1}), in that river (Ramani et al., 2014). The negative influence of metals on chub condition in our investigation is illustrated on F2 axis in Figure 3. This effect of metals was also implied by the observed severe damages in the gills of chub from the Zletovska and Kriva River (Barišić et al., 2015). Also, Zn and Mn in high concentrations may cause erythrocyte destruction (Tomova, Arnaudov and Velcheva, 2008; Sharma and Langar, 2014) and hemoglobin reduction (Kori-Siakpere and Ubogu, 2008; Sharma and Langar, 2014). It should be noted that Zn and Mn, did not show increased bioaccumulation in livers and gills, despite the high water contamination at the Zletovska River, indicating internal regulation of those elements (Dragun et al., 2019). On the other hand, Cd and Zn are shown to interact and change each others' absorption and distribution phases (El-Refaiy and Eissa, 2012). In addition, hemosiderin accumulation could be attributed to parasite infections (Dezfuli, Dezfuli, Manera, DePasquale, Pironi and Giari, 2017), but this seems unlikely in this case, as fish from Bregalnica and Kriva River had more parasites than fish from Zletovska River (Jordanova et al., 2016). As we mentioned previously, melanin was the most prominent pigment in spring season, but seems that the combinations in which melanin appears with other pigments were different in fish exposed to different pollutants. Namely, melanin is accompanied by lipochrome accumulation is associated with rivers in which organic matter was present in the water (the Bregalnica River and the Kriva River), whereas if the fish which were exposed to high concentrations of heavy metals (the Zletovska River), melanin was accompanied by accumulation of both lipochrome and hemosiderin. The accumulation of lipochrome can be considered as a normal process of aging (in the form of lipofuscin), but also as a result of tissue destruction due to oxidative stress (in the form of ceroid) (Yin, 1996; Terman and Brunk, 1998; Terman et al. 2007). Since the fish in our sample were of similar age (Table 1), differences in the accumulation of this pigment had to be a result of the destructive processes present in the fish from all three rivers. Taking this in consideration, it appears that the complex interaction of contaminants of the Kriva River with both organic and inorganic contaminants may be the reason of melanin and lipochrome increase in the Vardar chub MAs. As mentioned previously, heavy metals are the most likely candidates for causing oxidative tissue destruction, and although high concentrations of Cd were also detected in the Kriva River, the different mechanisms of metal interactions with fish may be an explanation of the different pigment accumulation. Thus, although the higher values registered for hemosiderin+lipochrome and hemosiderin+lipochrome+melanin laden MAs in the fish from the Zletovska River cannot be directly attributed to these processes, they may be offered as a credible explanation for pigment distribution differences in MAs of the fish spleen from the Zletovska River and the Kriva River.

Conversely, in the splenic MAs in the fish captured during the autumn season, lipochrome was a more dominant pigment, and it was accompanied by increased accumulation of hemosiderin in the fish exposed to heavy metals, and of melanin when fish were exposed to organic matter. This was revealed through hemosiderin accumulation in MAs in the fish from the Zletovska River which were exposed to even higher concentrations of Cd, Mn, and Zn in the autumn compared to the spring season, accompanied by reduced water level (Ramani et al. 2014). In fact, most of the parameters of MAs loaded with combinations of pigments containing hemosiderin were increased in the fish from the Zletovska River. In fish from the Kriva River, which were exposed to mild concentrations of heavy metals, but high concentrations of fecal bacteria (Ramani et al. 2014), lipochrome was the most abundant pigment, confirmed by the high values of MAs parameters. Significantly higher values were also detected for hemosiderin+lipochrome and hemosiderin+lipochrome+melanin laden MAs in the fish from this river, but with predominant accumulation of lipochrome in both combinations. Melanin was expected to be present in the spleen of the fish from the Kriva River, due to its suggested bactericide function in the fish (Wolke, George and Blazer, 1985). Presence of hemosiderin, on the other hand, could not been attributed to the influence of heavy metals as in the fish from the Zletovska River. Namely, concentrations of heavy metals in the water of the Kriva River in the autumn season were lower than in the spring season, and lower than the recommended environmental quality standards (Ramani et al., 2014). Still, the increased hemosiderin may be explained by the tendency of macrophages to retain iron as a protective mechanism from pathogenic bacteria (Belosevic et al. 2009; Grayfer, Hodgkinson and Belosevic, 2014), or by the excessive erythrocyte destruction due to infection (Agius and Roberts, 2003), which would be consistent with the high fecal contamination of the Kriva River water (Ramani et al., 2014). Other influences should not be ignored, such as the influence of parasites (Dezfuli, Dezfuli, Manera, DePasquale, Pironi and Giari, 2017), which were most prominent in the Kriva River (Jordanova et al. 2016).

Conclusions

Although all three pigments, melanin, lipochrome and hemosiderin, were present in MAs in Vardar chub spleens, lipochrome was the most abundant pigment for this species, especially in the autumn. Melanin accumulation was also seasonally dependant, with higher values observed in the spring, which was especially evident in the case of complex river water contamination with both heavy metals and organic matter. Both high metal pollution and fecal pollution of the river water were found to contribute to the accumulation of hemosiderin in MAs, the first one most likely as a result of erythrocyte destruction, and the second one probably as a result of iron retention due to bacterial and/or parasite infections. In the latter, hemosiderin often appears in the combination with lipochrome and melanin. However, it should be emphasized that MAs were considered to contain a combination of pigments, even if one of the pigments dominated, while the others appeared in small amounts. Therefore, these results should be viewed as a guide for further investigations, since to our knowledge the information about pigment content of MAs in relation to type of pollutant are very scarce.This study provided important information about the changes in the pigment content of MAs in the spleen of Vardar chub and can be used as a basis and guideline for the further studies of the differences in pigment accumulation in different seasons and under different pollution pressures, advisably under controlled laboratory conditions.

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References

Agius, C. (1979). The role of melano-macrophage centres in iron storage in normal and diseased fish. *Journal of Fish Diseases*, 2(4), 337-343.https://doi.org/10.1111/j.1365-2761.1979.tb00175.x

Agius, C. (1981). The effects of splenectomy and subsequent starvation on the storage of haemosiderin by the melano-macrophages of rainbow trout *Salmo gairdneri* Richardson. *Journal of Fish Biology*, 18(1), 41-44.https://doi.org/10.1111/j.1095-8649.1981.tb03757.x

Agius, C., & Agbede, S. A. (1984). An electron microscopical study on the genesis of lipofuscin, melanin and haemosiderin in the haemopoietic tissues of fish. *Journal of Fish Biology*, 24(4), 471-488.https://doi.org/10.1111/j.1095-8649.1984.tb04818.x

Agius, C.,& Roberts, R. J. (2003). Melano-macrophage centres and their role in fish pathology.*Journal of Fish Diseases*, 26(9), 499–509.doi: 10.1046/j.1365-2761.2003.00485.x

Barišić, J., Dragun, Z., Ramani, S., Marijić, V. F., Krasnići, N., Čož-Rakovac, R., Kostov, V., Rebok, K., & Jordanova, M. (2015). Evaluation of histopathological alterations in the gills of Vardar chub (*Squalius vardarensis* Karaman) as an indicator of river pollution. *Ecotoxicology and environmental safety*, *118*, 158-166.

Barst, B. D., Bridges, K., Korbas, M., Roberts, A. P., Van Kirk, K., McNeel, K., & Drevnick, P. E. (2015). The role of melano-macrophage aggregates in the storage of mercury and other metals: An example from yelloweye rockfish (*Sebastes ruberrimus*). *Environmental Toxicology and Chemistry*, 34(8), 1918-1925.doi:10.1002/etc.3009

Belosevic, M., Haddad, G., Walsh, J.G., Grayfer, L., Katzenback, B.A., Hanington, P.C., Neumann, N.F., & Stafford, J.L. (2009). Innate immunity of fish: antimicrobial responses of fish macrophages. In Zaccone, G., Meseguer, J., García-Ayala, A., & Kapoor, B.G. (Eds.), *Fish Defenses: Immunology* (Vol 1, pp. 145-184).Enfield, NH, USA: Science Publishers.

Blazer, V. S., Wolke, R. E., Brown, J., & Powell, C. A. (1987). Piscine macrophage aggregate parameters as health monitors: effect of age, sex, relative weight, season and site quality in largemouth bass (*Micropterus salmoides*). *Aquatic Toxicology*, 10(4), 199–215.https://doi.org/10.1016/0166-445X(87)90012-9

Dang, M., Nowell, C., Nguyen, T., Bach, L., Sonne, C., Nørregaard, R., Stride M., & Nowak, B. (2019). Characterisation and 3D structure of melanomacrophage centers in shorthorn sculpins (Myoxocephalus scorpius). *Tissue and Cell*, 57, 34-41.https://doi.org/10.1016/j.tice.2019.02.003

Dezfuli, B. S., Manera, M., DePasquale, J. A., Pironi, F., & Giari, L. (2017). Liver of the fish *Gymnotus inaequilabiatus* and nematode larvae infection: Histochemical features and expression of proliferative cell nuclear antigen. *Journal of fish diseases*, *40*(12), 1765-1774.https://doi.org/10.1111/jfd.12641

Dragun, Z., Tepić, N., Ramani, S., Krasnići, N., Filipović Marijić, V., Valić, D., Kapetanović, D., Erk, M., Rebok, K., Kostov, V., & Jordanova, M. (2019). Mining waste as a cause of increased bioaccumulation of highly toxic metals in liver and gills of Vardar chub (*Squalius vardarensis* Karaman, 1928). *Environmental Pollution*, 247, 564-576.doi:10.1016/j.envpol.2019.01.068

El-Refaiy, A. I., & Eissa, F. (2012). Protective effects of ascorbic acid and zinc against cadmium-induced histopathological, histochemical and cytogenetic changes in rats. *Comunicata Scientiae*, *3*(3), 162-180.doi: https://doi.org/10.14295/cs.v3i3.186

Fournie, J. W., Summers, J. K., Courtney, L. A., Engle, V. D., & Blazer, V. S. (2001). Utility of splenic macrophage aggregates as an indicator of fish exposure to degraded environments. *Journal of Aquatic Animal Health*, 13(2), 105-116.https://doi.org/10.1577/1548-8667(2001)013<0105:UOSMAA>2.0.CO;2

Freere, R. H., & Weibel, E. R. (1967). Stereologic techniques in microscopy. *Journal of the Royal Microscopical Society*, 87(1), 25-34.https://doi.org/10.1111/j.1365-2818.1967.tb04489.x

Giari, L., Manera, M., Simoni, E., & Dezfuli, B. S. (2007). Cellular alterations in different organs of European sea bass *Dicentrarchus labrax* (L.) exposed to cadmium. *Chemosphere*, *67*(6), 1171-1181.doi:10.1016/j.chemosphere.2006.10.061

Gil, F., & Pla, A. (2001). Biomarkers as biological indicators of xenobiotic exposure. *Journal of Applied Toxicology*, 21(4), 245-255.doi:10.1002/jat.769

Grayfer, L., Hodgkinson, J.W., & Belosevic, M. (2014).Antimicrobial responses of teleost phagocytes and innate immune evasion strategies of intracellular bacteria. *Developmental and Comparative Immunology*, 43(2), 223-242.doi:10.1016/j.dci.2013.08.003

Haaparanta, A., Valtonen, E. T., Hoffmann, R., & Holmes, J. (1996). Do macrophage centres in freshwater fishes reflect the differences in water quality?. *Aquatic Toxicology*, 34(3), 253-272.https://doi.org/10.1016/0166-445X(95)00042-3

James, R., Sampath, K., Jothilakshmi, S., Vasudhevan, I., & Thangarathinam, R. (2008). Effects of copper toxicity on growth, reproduction and metal accumulation in chosen ornamental fishes. *Ecohydrology and Hydrobiology*, 8(1), 89-97.

Jasim, B.M. (2008). Effects of prolonged exposure to cadmium on the hematopoietic organs in grass carp (*Ctenopharyngodonidella*, Cyprinidae). *Basrah Journal of Veterinary Research*, 7(2), 108-120.

Jordanova, M., Rocha, M. J., Rebok, K., & Rocha, E. (2011). Changes in the amount of kidney pigmented macrophage aggregates throughout the breeding cycle of female Ohrid trout, *Salmo letnica* Kar. (Teleostei, Salmonidae). *Microscopy research and technique*, 75(2), 176-181.doi: 10.1002/jemt.21040

Jordanova, M., Rebok, K., Naskovska, M., Kostov, V., & Rocha, E. (2016). Splenic Pigmented Macrophage Aggregates in Barbel (*Barbus peloponnesius*, Valenciennes, 1844) from River Bregalnica—Influences of Age, Sex and Season on a Pollution Biomarker. *Turkish Journal of Fisheries and Aquatic Sciences*, 16(4), 881-890.doi: 10.4194/1303-2712-v16_4_15

Jordanova, M., Rebok, K., Dragun, Z., Ramani, S., Ivanova, L., Kostov, V.,Valić, D., Krasnići, N., FilipovićMarijić, V., & Kapetanović, D. (2017). Effects of heavy metal pollution on pigmented macrophages in kidney of Vardar chub (*Squalius vardarensis* Karaman). *Microscopy research and technique*, *80*(8), 930-935.https://doi.org/10.1002/jemt.22884

Kapetanović, D., Valić, D., Smrzlić, I.V., Jordanova, M., Rebok, K., Ramani, S., & Cvetkovikj, A. (2014). Water quality of mining impacted rivers in the north-eastern Macedonia: II. Microbiological water quality of rivers Bregalnica, Zletovska and Kriva–Preliminary results.InDragun, Z. (Ed.) *Influence of active mines on freshwater ecosystems* (pp.9-10).Zagreb, Croatia,RuđerBošković Institute.

Kennedy, C.J. (2011). The Toxicology of Metals in Fishes.In Farrell, A.P. (Ed.) *Encyclopedia of fish Physiology* (Vol. 3, pp. 2061-2068).San Diego, Calif, USA: Academic Press. doi: 10.1016/B978-0-12-374553-8.00236-7

Kori-Siakpere, O.,& Ubogu, E. O. (2008).Sublethal haematological effects of zinc on the freshwater fish, *Heteroclarias sp*. (Osteichthyes: Clariidae). *African Journal of Biotechnology*, *7*(12). http://dx.doi.org/10.5897/AJB07.706

Kurtović, B., Teskeredžić, E., & Teskeredžić, Z. (2008). Histological comparison of spleen and kidney tissue from farmed and wild European sea bass.*Actaadriatica*, 49(2):147-154

Maceda-Veiga, A., Monroy, M., & de Sostoa, A. (2012). Metal bioaccumulation in the Mediterranean barbel (*Barbus meridionalis*) in a Mediterranean River receiving effluents from urban and industrial wastewater treatment plants. *Ecotoxicology and Environmental Safety*, 76, 93-101.doi:10.1016/j.ecoenv.2011.09.013

Manera, M., Sera, R., Isani, G., & Carpené, E. (2000). Macrophage aggregates in gilthead sea bream fed copper, iron and zinc enriched diets. *Journal of Fish Biology*, 57(2), 457-465.https://doi.org/10.1111/j.1095-8649.2000.tb02184.x

Matsche, M. A., Blazer, V. S., & Mazik, P. M. (2019). Comparisons of Stereological and Other Approaches for Quantifying Macrophage Aggregates in Piscine Spleens. *Journal of Aquatic Animal Health*, 1-21.doi: 10.1002/aah.10086

Meinelt, T., Krüger, R., Pietrock, M., Osten, R.,&Steinberg, C. (1997). Mercury pollution and macrophage centres in pike (*Esox lucius*) tissues. *Environmental Science and Pollution Research*, 4(1), 32-

36.doi:10.1007/BF02986262
Mela, M., Randi, M. A. F., Ventura, D. F., Carvalho, C. E. V., Pelletier, E. & Oliveira Ribeiro, C. A. (2007). Effects of dietary methylmercury on liver and kidney histology in the neotropical fish *Hopliasmalabaricus*. *Ecotoxicology and Environmental Safety*, 68(3), 426-435.doi:10.1016/j.ecoenv.2006.11.013
Mizuno, S., Misaka, N., Miyakoshi, Y., Takeuchi, K., & Kasahara, N. (2002). Effects of starvation on melano-macrophages in the kidney of masu salmon (Oncorhynchusmasou). *Aquaculture*, 209(1), 247-255.doi:10.1016/S0044-8486(01)00716-5
Patey, G., Couillard, C.M., Pierron, F., Baudrimont, M., & Couture, P. (2017). Biotransformation, antioxidant and histopathological biomarker responses to contaminants in European and American yellow eels from the Gironde and St. Lawrence estuaries. *Chemosphere*, 188, 292-303. doi:10.1016/j.chemosphere.2017.08.139
Price, M.H.H. (2013). *Sub-lethal metal toxicity effects on salmonids: a review* (Report No. 64). Skeena Wild Conservation Trust.Smithers, BC.
Ramani, S., Dragun, Z., Kapetanović, D., Kostov, V., Jordanova, M., Erk, M., & Hajrulai-Musliu, Z. (2014). Surface water characterization of three rivers in the lead/zinc mining region of northeastern Macedonia. *Archives of environmental contamination and toxicology*, 66(4), 514-528.doi: 10.1007/s00244-014-0012-z
Rebok, K., Jordanova, M., & Tavciovska-Vasileva, I. (2011). Spleen histology in the female Ohrid trout, *Salmo letnica* (Kar.)(Teleostei, Salmonidae) during the reproductive cycle. *Archives of Biological Sciences*, 63(4), 1023-1030.doi:10.2298/ABS1104023R
Rebok, K., Tavčiovska-Vasileva, I., & Jordanova, M. (2015). Do Spleen Macrophage Aggregates Undergo Season-Dependent Changes ? A Stereomicroscopic Investigation In Wild Ohrid Trout (*Salmo letnica* Kar.) *Contributions, Section of Natural, Mathematical and Biotechnical Sciences, MASA*, 36(1), 11–17.doi: 10.20903/csnmbs.masa.2015.36.1.61
Reddy, S. J. (2012).Cadmium effect on histo-biomarkers and melano-macrophage centers in liver and kidney of *Cyprinus carpio*. *World Journal of Fish and Marine Sciences*, 4(2), 179-184.doi: 10.5829/idosi.wjfms.2012.04.02.61178
Russo, R., Yanong, R. P., & Terrell, S. P. (2007). Preliminary morphometrics of spleen and kidney macrophage aggregates in clinically normal blue gourami *Trichogaster trichopterus* and freshwater angelfish *Pterophyllum scalare*. *Journal of aquatic animal health*, *19*(1), 60-67.doi: 10.1577/H05-023.1
Oliveira Ribeiro, C. A., Vollaire, Y., Sanchez-Chardi, A., & Roche, H. (2005). Bioaccumulation and the effects of organochlorine pesticides, PAH and heavy metals in the Eel (*Anguilla anguilla*) at the Camargue Nature Reserve, France. *Aquatic Toxicology,* 74(1), 53-69.doi:10.1016/j.aquatox.2005.04.008
Sevcikova, M., Modra, H., Slaninova, A., & Svobodova, Z. (2011). Metals as a cause of oxidative stress in fish: a review. *VeterinarniMedicina*, 56(11), 537-546.
Sharma, J., & Langer, S. (2014). Effect of manganese on haematological parameters of fish, Garragotylagotyla. *Journal of Entomology and Zoology Studies*, *2*(3), 77-81.
Skouras, A. (2002). *The use of piscine innate immune responses as indicators for environmental pollution in marine ecosystems* (Doctoral dissertation).Universität Hannover, Hannover, Germany.
Spasovski, O. (2011). Heavy and toxic metals and nutrients in separate places in the River Bregalnica, (Eastern Macedonia). *Annual Of The University Of Mining And Geology "St. Ivan Rilski"*,54, 118-120.
Stipaničev, D., Dragun, Z., Repec, S., Rebok, K., & Jordanova, M. (2017). Broad spectrum screening of 463 organic contaminants in rivers in Macedonia. *Ecotoxicology and Environmental Safety*, 135, 48-59. doi: 10.1016/j.ecoenv.2016.09.004
Suresh, N. (2009). Effect of cadmium chloride on liver, spleen and kidney melano macrophage centres in Tilapia mossambica. Journal of Environmental Biology, 30, 505–508.
Tchounwou, P.B., Yedjou, C.G., Patlolla, A.K., & Sutton, D.J. (2012). Heavy metal toxicity and the environment. *Experimentia Supplementum*, 101, 133-164.doi: 10.1007/978-3-7643-8340-4_6
Terman, A., & Brunk, U. T. (1998). Lipofuscin: mechanisms of formation and increase with age. *APMIS*, 106(1-6), 265-276.doi: 10.1111/j.1699-0463.1998.tb01346.x
Terman, A., Gustafsson, B., & Brunk, U. T. (2007). Autophagy, organelles and ageing. *The Journal of pathology*, 211(2), 134-143.https://doi.org/10.1002/path.2094
Tomova, E., Arnaudov, A., & Velcheva, I. (2008). Effects of zinc on morphology of erythrocytes and spleen in Carassius gibelio. *Journal of Environmental Biology*, 29(6), 897–902.

van der Oost, R., Beyer, J., & Vermeulen, N.P. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology*, 13(2), 57-149.DOI:10.1016/s1382-6689(02)00126-6

Vinodhini, R., & Narayanan, M. (2009). The impact of toxic heavy metals on the hematological parameters in common carp (Cyprinus carpio L.). *Iranian Journal of Environmental Health Science and Engineering*, 6(1), 23-28. Retrieved from http://www.bioline.org.br/pdf?se09005

Wolke, R. E., George, C. J., & Blazer, V. S. (1985). Pigmented macrophage accumulations (MMC; PMB): possible monitors of fish health. *Parasitology and Pathology of Marine Organisms of the WorldOcean*, 25, 93-97.

Wolke, R. E., Murchelano, R. A., Dickstein, C. D., & George, C. J. (1985). Preliminary evaluation of the use of macrophage aggregates (MA) as fish health monitors. *Bulletin of Environmental Contamination and Toxicology*, 35(1), 222-227.

Wolke, R.E. (1992). Piscine macrophage aggregates: a review. *Annual Review of Fish Diseases*. 2, 91-108.https://doi.org/10.1016/0959-8030(92)90058-6

Yin, D. (1996). Biochemical basis of lipofuscin, ceroid, and age pigment-like fluorophores. *Free Radical Biology and Medicine*, 21(6), 871-888.doi:10.1016/0891-5849(96)00175-x

Zapata, A., Chiba, A., & Varas, A. (1996). Cells and tissues of the immune system of fish. In Iwama, G., & Nakanishi, T.(Eds.),*The fish immune system: organism, pathogen, and environment* (Vol.15, pp.47-52). San Diego, California, USA: Academic Press.

Zelikoff, J.T. (1993). Metal pollution-induced immunomodulation in fish.*Annual Review of Fish Diseases*, 305-325.https://doi.org/10.1016/0959-8030(93)90041-9

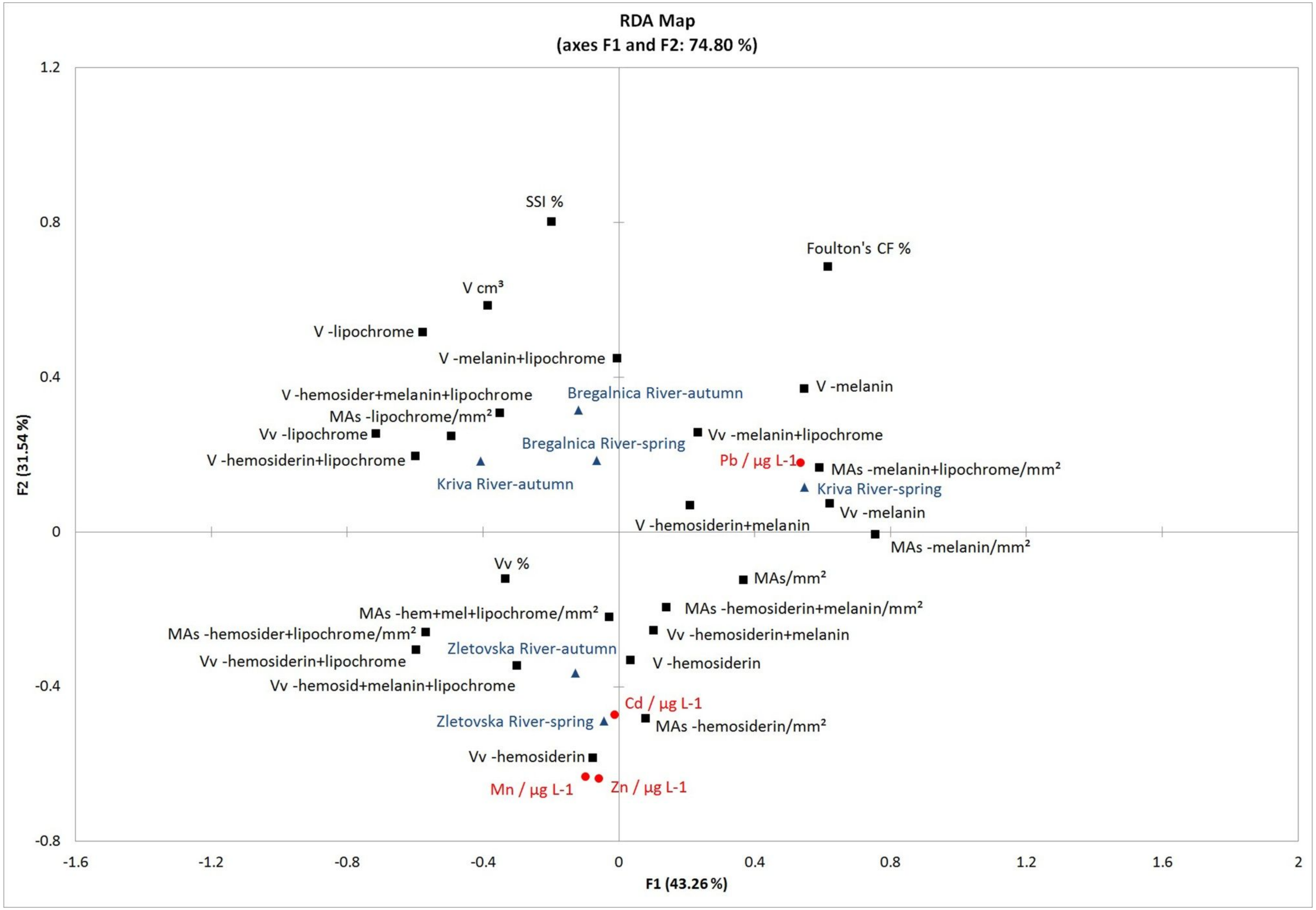
Zuasti., A., Jara, J.R., Ferrer, C., Solano, F. (1989).Occurrence of melanin granules and melanosynthesis in the kidney of *Sparus auratus*. *Pigment Cell Research*, 2(2), 93-99.doi:10.1111/j.1600-0749.1989.tb00168.x

Figure legends

Figure 1. Pigment content of MAs in fish spleen of chub collected from the Bregalnica River in the spring (a) and autumn season (b), the Kriva River in the spring (c) and autumn season (d) and from the Zletovska River in the spring (e) and autumn season (f). m-melanin, l-lipochrome, h-hemosiderin, Bar=10μm

Figure 2. Relative amounts of volumes and number of MAs with each pigment combination

Figure 3. Scatterplot representing factor scores from RDA (Factor 1: Factor 2) showing relationships between contaminants (red circles), locations in different seasons (blue triangles), and MAs response and chub condition (black squares) (a), and individual responses of fish collected in two seasons from the Bregalnica River (BA-autumn; BS-spring), the Kriva River (KA-autumn; KS-spring), and the Zletovska River (ZA-autumn; ZS-spring) (b)


RDA Map
(axes F1 and F2: 74.80 %)
F2 (31.54 %)
F1 (43.26 %)
1.2
0.8
0.4
0
-0.4
-0.8
-1.6
-1.2
-0.8
-0.4
0
0.4
0.8
1.2
1.6
2
SSI %
Foulton's CF %
V cm³
V -lipochrome
V -melanin+lipochrome
V -melanin
V -hemosider+melanin+lipochrome
Bregalnica River-autumn
MAs -lipochrome/mm²
Vv -lipochrome
Vv -melanin+lipochrome
Bregalnica River-spring
V -hemosiderin+lipochrome
Pb / µg L-1
MAs -melanin+lipochrome/mm²
Kriva River-autumn
Kriva River-spring
Vv -melanin
V -hemosiderin+melanin
MAs -melanin/mm²
Vv %
MAs/mm²
MAs -hemosiderin+melanin/mm²
MAs -hem+mel+lipochrome/mm²
MAs -hemosider+lipochrome/mm²
Vv -hemosiderin+melanin
Zletovska River-autumn
Vv -hemosiderin+lipochrome
V -hemosiderin
Vv -hemosid+melanin+lipochrome
Cd / µg L-1
Zletovska River-spring
MAs -hemosiderin/mm²
Vv -hemosiderin
Mn / µg L-1
Zn / µg L-1

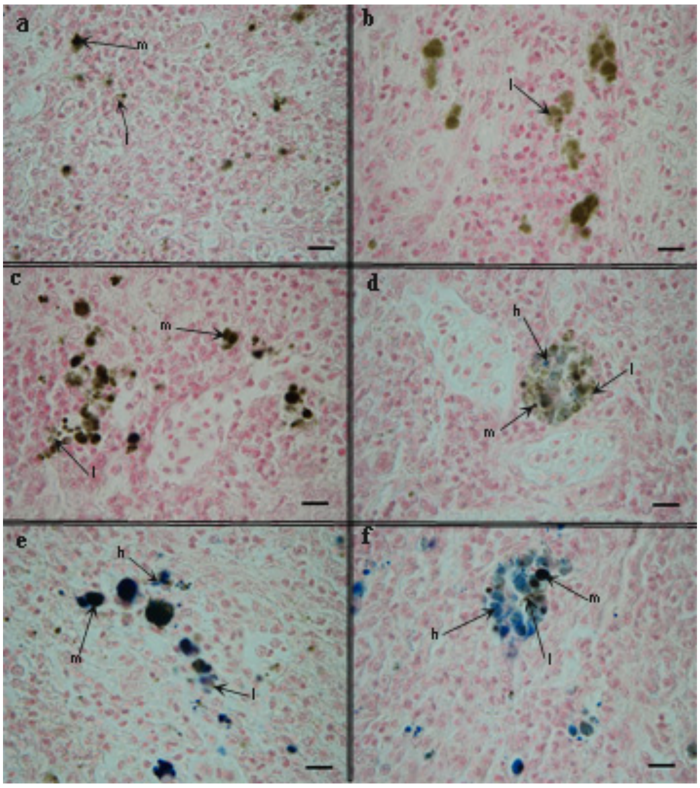


Figure 1

30x33mm (300 x 300 DPI)

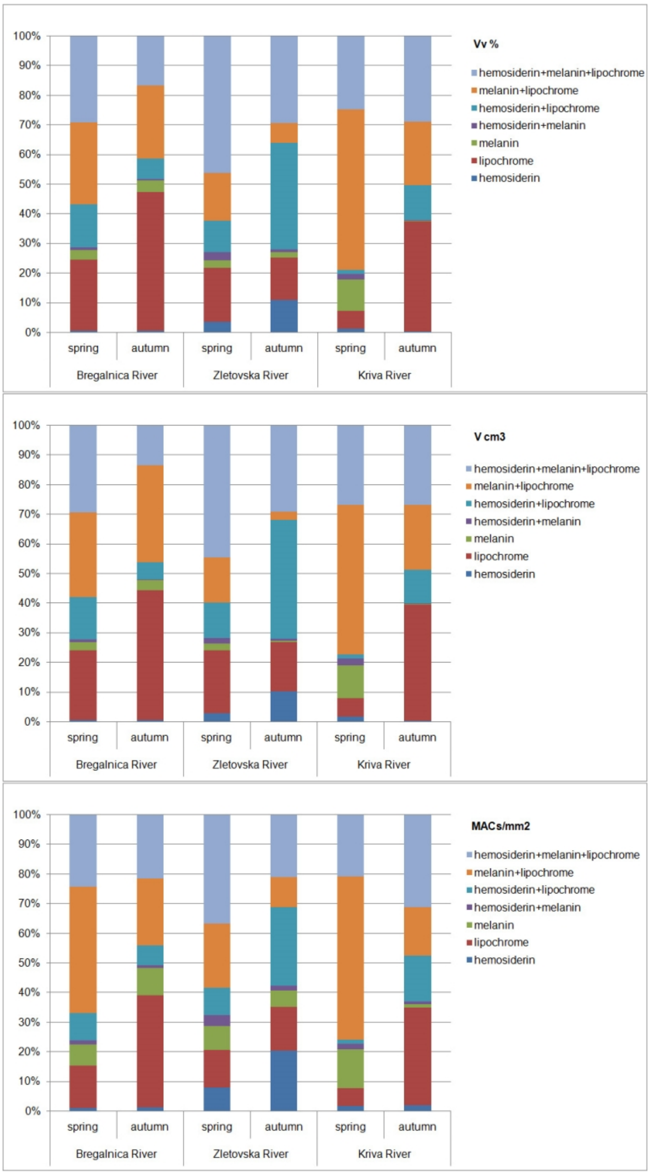


Figure 2

40x72mm (600 x 600 DPI)

Figure 3. Scatterplot representing factor scores from RDA (Factor 1: Factor 2) showing relationships between contaminants (red circles), locations in different seasons (blue triangles), and MAs response and chub condition (black squares) (a), and individual responses of fish collected in two seasons from the Bregalnica River (BA-autumn; BS-spring), the Kriva River (KA-autumn; KS-spring), and the Zletovska River (ZA-autumn; ZS-spring) (b)

285x419mm (300 x 300 DPI)

Table 1. Morphometric data for the examined Vardar chub: age, body weight (BW), Foulton's condition factor (Foulton's CF) and spleen weight (SW) of Vardar chub from three differently contaminated rivers, combined for both sexes, and presented separately for each season (spring and autumn). Results are presents as mean values, followed by coefficient of variation in brackets

	age (years)		BW (g)		Foulton's CF (%)		SW (g)		SSI (%)	
	spring	autumn	spring	autumn	spring	autumn	spring	autumn	spring	autumn
Bregalnica River	3,45 (0,30)	3,83 (0,24)	77,06 (0,74)	85,41 (0,64)	1,15 (0,09)	1,05 (0,05)	0,08 (0,57)	0,12 (0,60)	0,11 (0,39)	0,14 (0,29)
Zletovska River	3,06 (0,24)	2,50 (0,34)	29,76 (0,64)	32,09 (1,26)	0,98 (0,08)	0,90 (0,07)	0,02 (0,62)	0,02 (0,97)	0,08 (0,20)	0,07 (0,79)
Kriva River	3,31 (0,28)	3,75 (0,28)	72,99 (0,66)	60,78 (0,28)	1,14 (0,07)	0,96 (0,06)	0,07 (0,46)	0,09 (0,26)	0,11 (0,35)	0,15 (0,06)

Table 2. Relative (Vv) and total volumes (V) of pigmented macrophages (MACs) and number of MACs per mm^2 with different pigment content in the spleen of Vardar chub (age 2-5) from three differently contaminated rivers, combined for both sexes, and presented separately for each season (spring and autumn). Results are presents as mean values, followed by coefficient of variation in brackets

	spring						autumn					
	Vv		V		MACs/mm^2		Vv		V		MACs/mm^2	
BREGALNICA RIVER												
MACs-hemosiderin	0,02	(2,57)	0,001	(2,46)	1,25	(1,39)	0,01	(2,48)^{a}	0,001	(2,80)^{a}	0,80	(1,46)^{a}
MACs-lipochrome	0,66	(1,05)^{a}	0,05	(1,12)	18,72	(0,70)^{a}	0,77	(0,77)^{a}	0,11	(1,42)^{ab}	27,29	(0,48)
MACs-melanin	0,09	(1,62)^{a}	0,01	(1,90)^{a}	9,58	(1,78)^{a}	0,06	(1,65)	0,01	(1,65)	6,58	(1,71)^{a}
MACs-hemosiderin+melanin	0,03	(1,54)	0,002	(1,66)	1,83	(1,58)	0,01	(1,54)	0,001	(1,69)	0,68	(1,28)
MACs-hemosiderin+lipochrome	0,40	(1,10)^{Aa}	0,03	(1,19)^{a}	12,02	(0,89)^{a}	0,12	(1,20)^{Ba}	0,01	(1,50)^{a}	4,79	(0,58)^{a}
MACs-melanin+lipochrome	0,76	(1,06)^{ab}	0,06	(1,29)	56,29	(1,47)^{a}	0,40	(1,56)	0,08	(2,76)	16,29	(1,04)
MACs-hemosiderin+melanin+lipochrome	0,80	(1,17)^{ab}	0,06	(1,20)	31,73	(0,94)^{A}	0,27	(1,71)^{a}	0,03	(1,88)^{a}	15,35	(1,86)^{B}
ZLETOVSKA RIVER												
MACs-hemosiderin	0,10	(2,11)^{A}	0,002	(1,95)^{A}	9,54	(2,15)	0,38	(1,54)^{Bb}	0,008	(1,72)^{Bb}	22,06	(1,50)^{b}
MACs-lipochrome	0,50	(1,06)^{ab}	0,01	(1,47)	15,77	(0,69)	0,50	(1,35)^{a}	0,01	(2,16)^{b}	16,01	(0,97)
MACs-melanin	0,07	(1,94)^{a}	0,001	(1,55)^{a}	10,03	(2,81)^{a}	0,07	(0,98)	0,001	(0,63)	5,88	(0,85)
MACs-hemosiderin+melanin	0,08	(2,62)	0,001	(2,46)	4,40	(2,48)	0,03	(1,47)	0,001	(1,47)	1,78	(1,40)
MACs-hemosiderin+lipochrome	0,29	(1,20)^{Aa}	0,01	(1,55)^{b}	11,48	(1,37)^{a}	1,26	(1,33)^{Bb}	0,03	(1,46)^{ab}	28,81	(1,01)^{b}
MACs-melanin+lipochrome	0,45	(1,12)^{a}	0,01	(1,03)	26,69	(1,69)^{a}	0,24	(1,10)	0,002	(1,22)	11,07	(1,15)
MACs-hemosiderin+melanin+lipochrome	1,28	(1,13)^{a}	0,03	(1,30)	45,27	(0,93)	1,03	(1,25)^{ab}	0,02	(1,11)^{a}	22,81	(1,23)
KRIVA RIVER												
MACs-hemosiderin	0,03	(1,37)	0,002	(1,67)	3,25	(1,32)	0,01	(1,70)^{a}	0,001	(1,92)^{a}	1,39	(1,13)^{ab}
MACs-lipochrome	0,14	(1,24)^{Ab}	0,01	(1,08)^{A}	12,49	(1,91)^{Ab}	2,17	(1,03)^{Bb}	0,20	(1,16)^{Bb}	26,15	(0,59)^{B}
MACs-melanin	0,25	(1,07)^{Ab}	0,02	(0,91)^{Ab}	27,23	(1,40)^{Ab}	0,01	(1,20)^{B}	0,001	(1,15)^{B}	1,02	(1,24)^{B}
MACs-hemosiderin+melanin	0,04	(2,84)	0,004	(3,37)	3,59	(2,80)	0,01	(1,32)	0,001	(1,30)	0,71	(0,95)
MACs-hemosiderin+lipochrome	0,03	(1,23)^{Ab}	0,002	(1,23)^{Ab}	3,00	(1,37)^{Ab}	0,69	(0,86)^{Bb}	0,06	(0,92)^{Bb}	12,17	(0,58)^{Bab}
MACs-melanin+lipochrome	1,29	(1,22)^{b}	0,07	(1,08)	113,46	(1,27)^{Ab}	1,24	(1,05)	0,11	(1,22)	12,97	(0,75)^{B}
MACs-hemosiderin+melanin+lipochrome	0,59	(1,28)^{Ab}	0,04	(1,38)^{A}	42,98	(1,31)	1,69	(0,63)^{Bb}	0,14	(0,60)^{Bb}	24,63	(0,42)

†For each value, different small 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 Tukey test

Table 3. Standardized canonical coefficient and squared cosines extracted with redundancy analysis (RDA), the first showing the effect strength of each coefficient from the explanatory variables, and the later the contributions of variables on factors.

Standardized canonical coefficients:	F1	F2
Cd / µg L-1	-0.7159	-0.5161
Mn / µg L-1	0.2772	-0.2548
Pb / µg L-1	3.3626	0.0246
Zn / µg L-1	1.0061	-0.2473
Bregalnica River	0.5585	0.1052
Kriva River	-1.1110	0.1426
Zletovska River	0.4612	-0.2483
autumn	0.4285	0.1406
spring	-0.4285	-0.1406
Squared cosines (Response variables):		
Vv -hemosiderin	0.0134	0.8039
V -hemosiderin	0.0046	0.4198
MAs -hemosiderin/mm²	0.0205	0.7429
Vv -lipochrome	0.8752	0.1102
V -lipochrome	0.5223	0.4174
MAs -lipchrome/mm²	0.5889	0.1481
Vv -melanin	0.8759	0.0125
V -melanin	0.6297	0.2895
MAs -melanin/mm²	0.9307	0.0001
Vv -hemosiderin+melanin	0.1045	0.6460
V -hemosiderin+melanin	0.6810	0.0725
MAs -hemosiderin+melanin/mm²	0.2308	0.4495
Vv -hemosiderin+lipochrome	0.5690	0.1480
V -hemosiderin+lipochrome	0.6302	0.0676
MAs -hemosiderin+lipochrome/mm²	0.6812	0.1424
Vv -melanin+lipochrome	0.1617	0.1974
V -melanin+lipochrome	0.0001	0.8695
MAs –melanin+lipochrome/mm²	0.7758	0.0609
Vv -hemosiderin+melanin+lipochrome	0.2011	0.2668
V -hemosiderin+melanin+lipochrome	0.2989	0.2317
MAs -hemosiderin+melanin+lipochrome/mm²	0.0025	0.1517
Vv %	0.3130	0.0412
V cm³	0.2628	0.6076
MAs/mm²	0.5314	0.0607
Foulton's CF %	0.3287	0.4085
SSI %	0.0542	0.8967
Eigenvalues and percentages of inertia (RDA):		
Eigenvalue	2.2063	1.6084
Constrained inertia (%)	43.2628	31.5384
Cumulative %	43.2628	74.8012