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RESEARCH ARTICLE

Malondialdehyde concentrations in the intestine and gills of Vardar chub (*Squalius vardarensis* Karaman) as indicator of lipid peroxidation

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Abstract A lipid peroxidation product, malondialdehyde (MDA), was studied in Vardar chub (*Squalius vardarensis* Karaman) as an indicator of oxidative stress, using native fish from three rivers in northern Macedonia: the mining-impacted Zletovska and Kriva rivers and the agriculturally impacted Bregalnica River. MDA concentrations were measured in the intestine in the spring and autumn of 2012 and in the gills in autumn. The aims of the study were to establish the type of contamination which provokes a more pronounced MDA increase, as well as the organ which more reliably reflects the occurrence of oxidative stress. MDA levels in the intestine in spring amounted to 3.29–155.8 nmol g^{-1} and in autumn to 4.85–111.1 nmol g^{-1}, whereas MDA concentrations in the gills in autumn were 7.69–147.5 nmol g^{-1}. Stronger influence of organic contamination on development of oxidative stress was observed in both organs, as seen from higher median MDA concentrations in autumn in fish from the highly pesticide-contaminated Bregalnica River (gills 78.4 nmol g^{-1}; intestine 23.5 nmol g^{-1}) compared to the highly metal-contaminated Zletovska River (gills 15.9 nmol g^{-1}; intestine 17.4 nmol g^{-1}). The response of the gills to contamination was twice stronger than that of the intestine. The majority of fish from the pesticide-polluted river had increased MDA in the gills, in contrast to only sporadically increased MDA in the intestine. Our results indicated that development of oxidative stress strongly depends on the selected fish organ and that the gills seem to be a better choice for monitoring oxidative stress than the intestine, due to their continuous and direct exposure to polluted river water.

Keywords Gills · Intestine · Malondialdehyde · Metals · Pesticides · Vardar chub

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Introduction

Oxidative stress in organisms appears when the balance between oxidants and antioxidants is disrupted due to either inadequate antioxidant activity or excessive accumulation of the reactive oxygen species (ROS), or both, leading to oxidative damage (Scandalios 2005; Simonato et al. 2011). The possible oxidative damage refer to modified DNA, proteins and lipids, which lead to mitochondrial bioenergetics failure and, finally, to cell apoptosis or necrosis (Chuang 2010). A frequently used indicator of oxidative stress is lipid peroxidation, as an indicator of cell membrane damage by ROS (Valavanidis et al. 2006). The extent of lipid peroxidation depends on the relation between the production of oxidants and their removal and scavenging by antioxidant mechanisms (Jos et al. 2005). One of the lipid peroxidation products deriving from oxidative attack on cell membrane phospholipids and

circulating lipids is malondialdehyde (MDA), and its level directly reflects the degree of oxidative damage induced by contaminants (Banerjee et al. 1999). MDA has been considered as a reliable biomarker for lipid peroxidation (Valko et al. 2005) in both plants and animals (Li et al. 2015).

In nature, numerous xenobiotics have been described as causes of generation of free radicals and/or alteration of antioxidant enzyme systems which scavenge ROS (Stohs et al. 2000; Huang et al. 2007). According to numerous reports, in aquatic systems, antioxidant enzyme activities can be affected by heavy metals and pesticides, finally resulting in a certain degree of oxidative damage, such as lipid peroxidation, in aquatic organisms (Livingstone 2001; Huang et al. 2007). Specifically, lipid peroxidation has been suggested as one of the molecular mechanisms involved in pesticide-induced toxicity (Kehrer 1993). Pesticides, which are extensively used in intensive agricultural production and fish farms to control the pest population, can reach natural waters (Yonar et al. 2012) and thus also can affect non-target organisms, such as fish, which are of great economic importance to humans (Saravanan et al. 2010).

In northern Macedonia, severe water contamination of several rivers by both heavy metals (Ramani et al. 2014) and organic contaminants, including pesticides (Stipaničev et al. 2017), has been confirmed. Comprehensive study has been performed to establish if the high level of contamination with inorganic and organic compounds has caused toxic effects on native fish in those rivers. Vardar chub (*Squalius vardarensis* Karaman) has been selected as a bioindicator organism, being, as a fish species, placed at the top of the aquatic food chain and therefore mirroring the combination of the biotic and abiotic conditions in each particular aquatic environment (Chovanec et al. 2003; Dragun et al. 2007; Barišić et al. 2015). Furthermore, Vardar chub is a member of the fish genus *Squalius*, widespread in European freshwaters, therefore providing the possibility for comparison among distant geographical regions (Barišić et al. 2015). Toxic effects on the health of Vardar chub residing in the rivers of northern Macedonia were confirmed and were especially pronounced in the specimens from the mining-impacted Zletovska and Kriva rivers, and somewhat milder in the fish dwelling in the agriculturally impacted Bregalnica River, and were manifested as histopathological alterations in chub liver and gills (Barišić et al. 2015; Jordanova et al. 2016).

Reports on the real toxic effects, such as oxidative stress, as a result of exposure of aquatic organisms to contaminated water at the environmental level are rather scarce, and, therefore, it is recommendable to use field animals inhabiting polluted sites as bioindicator species for monitoring the environmental toxicity of polluted water (Huang et al. 2007). Accordingly, the aim of our study was to define if the known water contamination of rivers in northern Macedonia had also caused oxidative stress in native fish from these rivers. By this approach, we were, thus, able to obtain information on the current health status of fish from rivers in northern Macedonia and also to broaden the knowledge and understanding of development of oxidative stress under realistic exposure conditions. To accomplish this, we have selected two target organs of Vardar chub, the gills and intestine. Fish gills are particularly sensitive to water quality, constituting the first target of pollutants, due to their anatomic location, direct contact with water and quick absorption (Pandey et al. 2008). On the other hand, intestinal tissue reflects dietborne pollution impact, which in addition to waterborne, might be of primary importance in causing toxicological response (Filipović Marijić and Raspor 2012). Specifically, we aimed to compare the development of oxidative stress between (1) fish from metal- and pesticide-contaminated waters, (2) two target organs and (3) two seasons, spring and autumn.

Materials and methods

Study period and area

The study of oxidative stress in freshwater fish species, Vardar chub (*S. vardarensis*), was conducted in three rivers in northeastern Macedonia in two seasons of 2012 (May/June as representative of spring and October as representative of autumn). One studied river was characterized by agricultural impact (Bregalnica) and the other two by different degrees of mining influence (Zletovska River and Kriva River).

The Bregalnica River is the longest left tributary of the Vardar River, the principal river in Macedonia. Bregalnica has a length of 225 km and a catchment area of 4307 km^2, and its water discharge in 2012 ranged from 1.24 to 66.3 $m^3\ s^{-1}$ (Ramani et al. 2014). The area along the course of the Bregalnica River, including the regions of Kočani, Štip, Vinica and Blatec, is known as a rice production core of the Republic of Macedonia (Andreevska et al. 2013). The sampling point at this river was selected at the site known as Kežovica (N 41° 43.57′, E 22° 10.27′), which was located approximately 35 km downstream from the mouth of the Zletovska River into the Bregalnica River and 3 km downstream from Štip, the largest town in the eastern part of Macedonia (population in 2002, 47,796). This site was, therefore, influenced not only by runoff from rice fields but also by sewage and household water discharges, industrial facilities and farms of the town (textile factories, meat processing industry, factory for production of edible oil, poultry farm, pig farm) (Spasovski 2011; Rebok 2013).

The Zletovska River is one of the most polluted tributaries of the Bregalnica River (Dolenec et al. 2005). It is 56 km long, it has a catchment area of 460 km^2, and its water discharge in 2012 ranged from 0.167 to 26.55 $m^3\ s^{-1}$ (Ramani et al. 2014). This river receives the effluents from the active Pb/Zn mine

Zletovo (Alderton et al. 2005; Dolenec et al. 2005). The sampling point at the Zletovska River (N 40° 58.54″, E 21° 39.45″) was located 5–6 km downstream from the Zletovo mine and 15 km downstream from the town Probištip (population in 2002, 10,826). In Probištip, there is also a battery factory, as a potential source of water contamination (Spasovski and Dambov 2009).

The Kriva River is the longest tributary of the river Pčinja, which is a left tributary of the Vardar River. The length of the Kriva River is 78.7 km, it has a catchment area of 968 km^2, and its water discharge in 2012 ranged from 0.08 to 8.42 $m^3\ s^{-1}$ (Ramani et al. 2014). The sampling point at the Kriva River (N 42° 11.39″, E 22° 18.34″) was located 15 km downstream from the active Pb/Zn mine Toranica. In addition to mining, the Kriva River is also impacted by agricultural runoff, since it flows through cultivated land (gardens, orchards).

The map of the study area, detailed physico-chemical and microbiological characterization, and data on dissolved concentrations of numerous metals and metalloids in the surface water samples of these three rivers, which were taken simultaneously with chub sampling, were published by Ramani et al. (2014). The concentrations of organic contaminants in the surface river water at the described sampling sites of the three studied rivers were determined afterwards, in May of 2015 (Stipaničev et al. 2017).

Fish sampling

A total of 158 specimens of Vardar chub (*S. vardarensis*) was sampled during this investigation. In the spring of 2012, a total of 90 specimens was sampled, i.e. 30 specimens per river. In the autumn of 2012, a total of 68 specimens was sampled, i.e. 30 specimens from the Bregalnica River, 26 from the Kriva River and 12 from the Zletovska River. Fish sampling was performed by electrofishing (electrofisher Samus 725G) according to relevant standards (CEN EN 14011:2003). Captured fish were kept alive in an opaque plastic tank with aerated river water until further processing in the laboratory. Individual fish were anaesthetized with clove oil (Sigma-Aldrich, USA). Total length and total mass were measured, and the Fulton condition indices (FCI) were calculated in accordance with Rätz and Lloret (2003). After fish sacrifice, the posterior intestine, gills and gonads were dissected, and their masses measured. The intestine and gills were stored in a refrigerator at −80 °C until further analyses, whereas the gonads were put in Bouin's fixative (Merck, Germany) for histological determination of fish sex. Gonadosomatic indices (GSI, %) were calculated as ratios between gonad masses and total body masses (TM, g), multiplied by 100 (Şaşi 2004). All sampled fish were used for comprehensive assessment of the consequences of metal exposure on Vardar chub; that was the reason why MDA analyses were not performed on the complete set of samples, but only on the samples that remained after determination of metals and metallothioneins and histopathology examination of chub organs (Barišić et al. 2015; Jordanova et al. 2016). The biometric characteristics and number of specimens of Vardar chub analysed for MDA per site and per season are given in Table 1. Due to small sample volumes obtained for the gills, MDA values for that organ were measured only for the autumn season.

Isolation of soluble tissue fractions for MDA determination

The samples of the posterior intestine and of the gills were first cut into small pieces. Then cooled homogenization buffer was added (w/v 1:5). The applied homogenization buffer was 100 mM Tris-HCl/base (Merck, Germany, pH 8.1 at 4 °C) supplemented only with a reducing agent (1 mM dithiothreitol, Sigma, USA), in the case of the gills (Dragun et al. 2007), and additionally with 0.5 mM phenylmethylsulfonyl fluoride (PMSF, Sigma, USA) and 0.006 mM leupeptin (Sigma, USA) as protease inhibitors, in the case of the intestine (Filipović Marijić and Raspor 2010). The next step was sample homogenization by ten strokes of the Potter-Elvehjem homogenizer (Glas-Col, USA) in an ice-cooled tube at 6000 rpm. The homogenates were centrifuged in the Avanti J-E centrifuge (Beckman Coulter, USA) at 3000×*g* for 10 min at 4 °C, which was a procedure adapted from Botsoglou et al. (1994). Supernatants (S3) obtained after centrifugation were separated and stored at −80 °C for subsequent MDA analyses.

MDA measurement

The levels of MDA were measured spectrophotometrically by a reaction of MDA with 2-thiobarbituric acid (TBA) at low pH and high temperature, which produces a pink, fluorescent product with maximum absorbance at a wavelength of 535 nm. The procedure was adapted from Botsoglou et al. (1994) and Lovrić et al. (2008). In the first step, aliquots of tissue supernatants (S3, 250 μL) were transferred in duplicate into 1.5-mL Eppendorf® vials placed on ice and followed by addition of 500 μL of mixture of 1% butylated hydroxytoluene (BHT, Sigma-Aldrich, USA) dissolved in ethanol (CARLO ERBA Reagents, Italy) and 10% trichloroacetic acid (TCA, Kemika, Croatia) dissolved in Milli-Q water (BHT/TCA = 1:100). The obtained mixtures were kept in a refrigerator at 4 °C for 15 min and then centrifuged in the Biofuge Fresco centrifuge (Kendro, USA) at 4000×*g* for 15 min at 4 °C. Aliquots of 750 μL of the deproteinized supernatants were transferred into 1.5-mL Eppendorf® vials, and in each vial, 500 μL of TBA (Alfa Aesar, Germany) dissolved in Milli-Q water to a concentration of 0.667% was added. The vials were then heated at 100 °C for 30 min. After cooling, aliquots of 200 μL were transferred into microplate wells and

Table 1 Biometric characteristics of Vardar chub (*Squalius vardarensis*) specimens used for analysis of MDA in the intestine in the spring of 2012. Total chub masses, Fulton condition indices (FCI) and gonadosomatic indices (GSI) are presented as medians, with minimums and maximums within brackets. Sex is presented as the number of female and male specimens within the analysed group

		Bregalnica	Zletovska	Kriva
Intestine	*n*	25	16	21
Spring 2012	Total chub mass (g)*	62.9 (13.9–307.9)[a]	28.9 (13.0–106.7)[b]	68.2 (32.7–231.5)[a]
	FCI ((g × 100) cm^{-3})*	1.15 (1.02–1.39)[a]	1.00 (0.89–1.10)[b]	1.16 (0.96–1.30)[a]
	GSI (%)	6.27 (1.14–11.97)[a]	8.18 (1.96–12.95)[a,b]	9.12 (5.17–18.25)[b]
	Sex (F/M)	11/14	10/6	8/13
Intestine	*n*	29	8	20
Autumn 2012	Total chub mass (g)*	71.6 (39.7–281.9)[a]	23.5 (6.1–107.2)[b]	30.0 (10.9–95.3)[b]
	FCI ((g × 100) cm^{-3})*	1.06 (0.91–1.20)[a]	0.86 (0.80–0.97)[b]	0.95 (0.90–1.13)[b]
	GSI (%)*	1.57 (0.73–3.97)[a,b]	4.74 (0.79–6.30)[a]	1.39 (0.67–5.27)[b]
	Sex (F/M)	16/13	6/2	5/15
Gills	*n*	25	2	7
Autumn 2012	Total chub mass (g)*	88.7 (42.1–281.9)	39.2 (27.1–51.3)	60.1 (45.5–95.3)
	FCI ((g × 100) cm^{-3})*	1.07 (0.91–1.20)[a]	0.83 (0.80–0.85)[b]	0.95 (0.90–1.07)[b]
	GSI (%)*	1.67 (0.73–3.97)	6.23 (6.16–6.30)	1.44 (1.20–5.27)
	Sex (F/M)	16/9	2/0	3/4

*According to the Kruskal-Wallis test, there was a statistically significant difference in the measured parameter between the three rivers (for the intestine $p < 0.01$, for the gills $p \leq 0.05$)

[a,b] According to post hoc Dunn's test, the rivers assigned different letters had significantly different values of the measured parameter ($p < 0.05$)

absorbance was read at 535-nm wavelength using the spectrophotometer/fluorometer microplate reader Infinite M200 (Tecan, Switzerland). The calibration curve was constructed using nine different concentrations (1–50 nmol mL^{-1}) of MDA (Aldrich, USA) dissolved in 1 N HCl (Kemika, Croatia). Homogenization buffer (100 mM Tris-HCl/base, Merck, Germany) was used as a blank sample. The results were obtained as nanomoles of MDA per millilitre of supernatant (S3) and finally expressed as nanomoles of MDA per gram of wet tissue mass. Limits of detection (LOD) and quantification (LOQ) for MDA determination were calculated using ten consecutive MDA measurements in the blank sample. LOD was calculated as the sum of the obtained standard deviation multiplied by 3 and the mean value of the blank, whereas LOQ was calculated as the sum of the obtained standard deviation multiplied by 10 and the mean value of the blank. LOD and LOQ amounted to 0.260 nmol mL^{-1} (1.56 nmol g^{-1}) and 0.740 nmol mL^{-1} (4.44 nmol g^{-1}), respectively.

Data processing and statistical analyses

The statistical program SigmaPlot 11.0 for Windows was applied for creation of graphs and statistical analyses. Due to the uneven number of data in the study groups from the three rivers, between-site comparisons were performed by Kruskal-Wallis one-way analysis of variance on ranks (levels of significance indicated in Fig. 1 and Table 1) with post hoc Dunn's test (level of significance set at $p < 0.05$). Between-site comparisons were performed for MDA concentrations (Fig. 1), total mass, FCI and GSI (Table 1), separately for each target organ and each season. Three multiple linear regression models for MDA concentrations were created (Table 2), separately for each organ and each season, by the use of a backward stepwise regression procedure on standardized values. The models included MDA as a dependent variable and six independent variables: three continuous (TM, FCI and GSI) and three binary (sex, high metal pollution and high pesticide pollution) variables. Differences in the intestinal MDA concentrations between males and females (Table 3), as well as between two seasons (Table 4), and differences in MDA values between two target organs (Table 5) were tested by two-way analysis of variance and by pairwise comparisons using the Holm-Sidak method, with the sampling site tested as a second factor of influence (*p* values are indicated in Tables 3, 4 and 5).

Results and discussion

Assessment of oxidative stress in Vardar chub (*S. vardarensis*) was performed in three differently contaminated rivers. Bregalnica was chosen as an agriculturally impacted river, and although analysis of water contamination with pesticides was not performed at the moment of chub sampling, subsequent analysis performed in May of 2015 confirmed high pesticide pollution of this river (Stipaničev et al. 2017). Total concentrations of all analysed pesticides in the surface water of the Bregalnica River at the sampling site Kežovica, where chub were previously sampled, amounted to 1294 ng L^{-1},

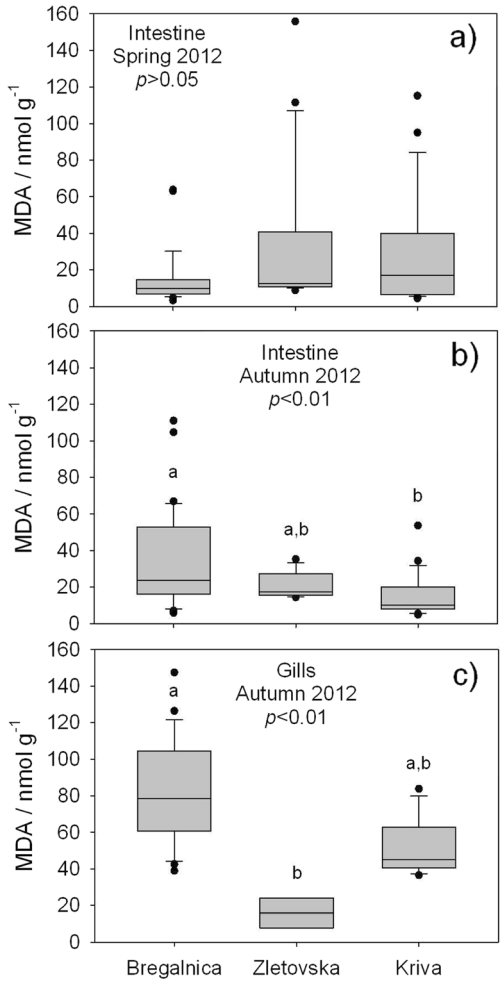

Fig. 1 Malondialdehyde concentrations (nmol g^{-1}) in two selected organs of Vardar chub (*Squalius vardarensis*) caught in three rivers in Macedonia (Bregalnica, Zletovska, and Kriva) in two seasons of 2012. **a** MDA in chub intestine in spring 2012. **b** MDA in chub intestine in autumn 2012. **c** MDA in chub gills in autumn 2012. The results are presented as *box plots*. The *boundaries of box plot* indicate 25th and 75th percentiles; a *line within the box* marks the median value; *whiskers above and below the box* indicate 10th and 90th percentiles, whereas the *black dots* present all outliers. Statistically significant differences between sites are indicated with *different letters* (*a*, *b*), based on Kruskal-Wallis one-way analysis of variance on ranks (levels of significance indicated in the figure) with post hoc Dunn's test (level of significance set at $p < 0.05$). Number of samples per site/per season is indicated in Table 1

compared to 184.5 and 296.5 ng L^{-1} in the Zletovska and the Kriva River, respectively (Stipaničev et al. 2017). The Zletovska River was chosen as a mining-impacted river, with extremely high concentrations of numerous metals (Cd, Co, Cs, Cu, Li, Mn, Ni, Rb, Sn, Sr, Tl and Zn) measured in its water precisely at the time of chub samplings and with especially high concentrations of Cd, Zn and Mn in the period of low water level in October of 2012 (Ramani et al. 2014). And, finally, the Kriva River was chosen as a river that merges these two unfavourable effects in a moderate degree, receiving both agricultural runoff and mining drainage but less pronounced compared to either the Bregalnica or Zletovska River (Ramani et al. 2014; Stipaničev et al. 2017). The sampling sites were selected according to general knowledge, acquired under laboratory conditions of acute exposure, that both individual organic and metallic contaminants could cause oxidative damage in aquatic organisms (Huang et al. 2007). However, it is often the case in natural ecosystems that water is simultaneously contaminated by both organic and inorganic contaminants, which is why the exact source of the observed oxidative stress cannot be discerned without a doubt. Such a case was reported for the gills of the African catfish (*Clarias gariepinus*), where increased MDA concentrations were observed after fish exposure to bridge runoff, containing not only high metal concentrations but also abundance of petroleum products (Amaeze et al. 2015). Therefore, our study on rivers distinctively contaminated by either metals or organic contaminants enabled hypothesizing about the type of contamination that causes a higher degree of oxidative stress.

Further on, our study was performed on two target organs, the intestine and gills, both being organs of contaminant uptake, only through different modes. Moreover, it was done on chub of different biometric characteristics, specifically of different sex, condition, and size, which reflects chub age and nutrition. And finally, intestinal tissue was analysed in two seasons, spring and autumn, therefore allowing the comparison of MDA response in fish of different metabolic rate and nutritional status (Table 1). Applying statistical analysis on such a heterogeneous data set, it was possible to further hypothesize about the uptake organ that is more susceptible to oxidative stress, as well as to discuss the possible association of fish physiology with MDA levels in the selected chub organs.

MDA values in chub intestine—spring and autumn of 2012

If chub from all three rivers were considered together, MDA values measured in the intestine in the spring period ranged from 3.29 to 155.8 nmol g^{-1}. The multiple linear regression model for MDA values in chub intestine in the spring period revealed high metal pollution as the main, statistically significant, effect (Table 2); however, only 5% of MDA variability was explained by this model (adjusted $R^2 = 0.049$). Accordingly, although MDA values were somewhat higher in the intestine of chub from the two mining-impacted rivers, Zletovska (median 12.6 nmol g^{-1}) and Kriva (median 17.0 nmol g^{-1}) compared to that of chub from the agriculturally impacted Bregalnica River (median 9.76 nmol g^{-1}) (Fig. 1a), differences between sites were not statistically significant. Moreover, within-site variability, which could not be associated with different levels of exposure to contaminants, was much higher (relative standard deviations (RSD) 103–126%, depending on the river) than between-site variability (RSD 37%), indicating that some other unaccountable factors

Table 2 Multiple regression models for MDA concentrations in chub intestine in spring and autumn, as well as for MDA concentrations in chub gills in the autumn of 2012

Models[a]	Effects	Coefficients	Squares	*p*
Model for MDA in intestine (spring)	High metal pollution	0.513	0.263	0.046
$R = 0.254$; adjusted $R^2 = 0.049$;				
$p = 0.046$; $n = 62$				
Model for MDA in intestine (autumn)	Sex	−0.689	0.475	0.005
$R = 0.512$; adjusted $R^2 = 0.235$;	High pesticide pollution	0.646	0.417	0.008
$p = <0.001$; $n = 57$				
Model for MDA in gills (autumn)	High pesticide pollution	1.173	1.376	0.001
$R = 0.580$; adjusted $R^2 = 0.293$;	Sex	0.585	0.342	0.058
$p = 0.002$; $n = 34$				

[a] The models included MDA as a dependent variable and six independent variables, three continuous (total chub mass (TM), Fulton condition index (FCI) and gonadosomatic index (GSI)) and three binary (sex, high metal pollution and high pesticide pollution). The final models were obtained by the use of backward stepwise regression on standardized values

probably had more pronounced influence on intestinal MDA during the spring period.

In the autumn period, MDA values measured in the intestine of all analysed chub ranged from 4.85 to 111.1 nmol g^{-1}. The multiple linear regression model for MDA values in chub intestine in the autumn period revealed comparable, statistically significant, influence of two effects on MDA levels (comparable squares, Table 2), chub sex and high pesticide pollution. Furthermore, the model obtained for the intestine in autumn explained a much higher percentage of MDA variability (24%; adjusted $R^2 = 0.235$).

Accordingly, the spatial distribution obtained in the autumn period was opposite to the one observed in spring: higher MDA values were now measured in the intestine of chub from the river highly polluted by pesticides, i.e. from the Bregalnica River (median 23.5 nmol g^{-1}), compared to the mining-impacted rivers (Zletovska, median 17.4 nmol g^{-1}; Kriva, median 9.99 nmol g^{-1}); the difference between the Bregalnica and the Kriva River was even statistically significant (Fig. 1b). However, similar to spring, within-site variability was again generally higher (RSD 36–80%, depending on the river) than between-site variability (RSD 41%).

To further evaluate sex differences in MDA levels in the autumn period, two-way ANOVA was performed, with sex and river as factors of influence (Table 3). Looking at all three rivers together, MDA was 75% higher in females than in males, and the difference was statistically significant. Interaction between sex and river was not significant, meaning that sex differences did not depend on the river from which chub were sampled. Even if each river was analysed separately, female specimens had higher levels of MDA than males (50–100%), but the difference was statistically significant only in Bregalnica (100% higher values in females). Contrary to autumn, sex differences in MDA in chub intestine were not established by two-way ANOVA for the spring sampling, which was in accordance with the multiple linear regression model for intestinal MDA in spring.

As already clearly observed from the models obtained for intestinal MDA in two seasons, there were some distinctive differences between spring and autumn regarding development of oxidative stress. If MDA values of all three rivers were compared between two seasons, there was no statistically

Table 3 Differences in intestinal MDA concentrations between males and females of Vardar chub in the autumn of 2012 tested by two-way analysis of variance and pairwise comparison using the Holm-Sidak method. The results are presented as least square means with assigned standard error

	Intestinal MDA in autumn (nmol g^{-1})		*p*
	Males	Females	
n	30	27	
All three rivers	16.84 ± 5.37	29.50 ± 4.38	0.074
Interaction, sex × river			0.404
Bregalnica	21.82 ± 5.57	44.58 ± 5.02	0.004
Zletovska	14.75 ± 14.19	23.39 ± 8.20	0.600
Kriva	13.97 ± 5.18	20.52 ± 8.98	0.530

Table 4 Seasonal differences in MDA concentrations in the intestine of Vardar chub (*Squalius vardarensis*) tested by two-way analysis of variance and pairwise comparison using the Holm-Sidak method. The results are presented as least square means with assigned standard error

	Intestinal MDA (nmol g^{-1})		*p*
	Spring, 2012	Autumn, 2012	
n	62	57	
All three rivers	26.26 ± 3.41	23.74 ± 4.03	0.634
Interaction, season × river			0.006
Bregalnica	15.42 ± 5.29	34.38 ± 4.91	0.010
Zletovska	34.40 ± 6.61	21.23 ± 9.34	0.252
Kriva	28.95 ± 5.77	15.60 ± 5.91	0.109

Table 5 Differences in MDA concentrations between the two target organs of Vardar chub (*Squalius vardarensis*), intestine and gills, in the autumn of 2012, tested by two-way analysis of variance and pairwise comparison using the Holm-Sidak method. The results are presented as least square means with assigned standard error. This comparison was performed on 32 chub, which were analysed for both intestinal and gill MDA

	MDA in autumn (nmol g^{-1})			p
	Intestine	Gills	Standard error	
All three rivers	27.78	50.65	7.37	0.032
Interaction, organ × river				0.216
Bregalnica	39.21	81.21	5.36	<0.001
Zletovska	21.30	15.87	18.57	0.837
Kriva	22.82	54.88	10.72	0.039

significant difference between them (Table 4). However, if seasonal differences were tested for each river separately, differences corresponded well to the obtained models for these two seasons which indicated increased MDA concentrations in the two mining-impacted rivers in spring as opposed to increased values in the agriculturally impacted Bregalnica River in autumn. Accordingly, in the Bregalnica River, twice higher values were obtained in the autumn period compared to spring, and the difference was statistically significant (Table 4). On the contrary, 60–85% higher MDA values in the Zletovska and the Kriva River were obtained in the spring period compared to autumn, but the differences were not statistically significant (Table 4). The reason why, despite high differences between mean values, these differences were not statistically significant can be found in rather high MDA variability within sites, which was observed in both seasons and which indicated that MDA values at specific sites were increased only sporadically, and not in all analysed specimens. Furthermore, it is interesting that the MDA level was increased in the metal-contaminated rivers in the spring period, and not in autumn, whereas exposure to metals was much higher in autumn during the extremely low water level in the Zletovska River than in spring; for example, the concentration of Cd in that river increased up to 2.0 μg L^{-1} and Zn up to 1.5 mg L^{-1}, compared to much lower concentrations in spring (Cd 270 ng L^{-1}; Zn 197 μg L^{-1}; Ramani et al. 2014). Therefore, our assumption is that sporadically increased intestinal MDA levels in chub from the metal-contaminated rivers in spring were probably in some way associated with chub physiological processes, such as more intense feeding in spring compared to autumn and depended on the feeding intensity of each individual fish. The ability of environmental factors, such as temperature, dissolved oxygen and food availability, to affect oxidative stress responses via their influence on metabolism and reproduction was previously reported (Sheehan and Power 1999; Danabas et al. 2015). Interaction of the antioxidant defence system with food supply was further described by Khessiba et al. (2005). On the other hand, autumn increase in intestinal MDA in the pesticide-contaminated Bregalnica River could possibly be associated with the fact that the typical period of pesticide application occurs in the period from April to July (Laganà et al. 2002; Papadakis et al. 2015), whereas spring fish sampling in the Bregalnica River in our study was performed in early May and therefore possibly completed before pesticides were even applied. It could also be presumed that the effect of pesticides on development of oxidative stress in the intestine was more pronounced than that of metals, considering that intestinal MDA levels in the pesticide-contaminated river were increased in the period of generally less active feeding, whereas during that same period, the MDA level was not increased in the Zletovska River, which was at that point characterized by extremely high concentrations of several metals in the surface water. High MDA increase, amounting to 119%, was also found in the intestine of carp (*Cyprinus carpio*) from the region of the Yellow River polluted by organic contaminants (phenols and oils) and unionized ammonia (Huang et al. 2007).

MDA values in chub gills—autumn of 2012

In the autumn period, MDA values measured in the gills of all analysed chub ranged from 7.69 to 147.5 nmol g^{-1}. They were somewhat lower compared to MDA values in the gills of another cyprinid fish, Tigris scraper (*Capoeta umbla*), from Uzuncayir Dam Lake in Turkey, which were also measured in autumn and ranged from 26 to 346 nmol g^{-1} (Danabas et al. 2015). The same as observed for intestinal MDA in autumn, the multiple linear regression model for MDA values in chub gills (Table 2) revealed their association with two effects, high pesticide pollution and sex. However, in the case of the gills, sex influence presented less pronounced effect (four times weaker than pesticide pollution according to squares of coefficients, Table 2) and less significant (p = 0.058 for sex and <0.001 for pesticide pollution, Table 2). This was further confirmed by two-way ANOVA, which indicated opposite sex dependence in the gills compared to the intestine, with higher MDA levels in males (least squares mean 75.1 nmol g^{-1}) than in females (least squares mean 57.8 nmol g^{-1}), but not statistically significant. Of all three models in this study, the model obtained for the gills in autumn explained the highest percentage of MDA variability (29%; adjusted R^2 = 0.293), indicating that a higher proportion of gill than intestinal MDA could be associated with water pollution. It was further confirmed by the highest between-site variability obtained precisely for gill MDA (RSD 65%).

Accordingly, spatial distribution of MDA in chub gills in the autumn period (Fig. 1c) reflected the known pesticide pollution of surface water of the studied rivers, i.e. the highest

MDA level was measured in the Bregalnica River (median 78.4 nmol g^{-1}), which is the most polluted with pesticides (Stipaničev et al. 2017), followed by the Kriva River (median 45.0 nmol g^{-1}), which is also somewhat influenced by agricultural runoff, and the lowest level was found in the Zletovska River (median 15.9 nmol g^{-1}), which is highly contaminated by metals, but not by pesticides. The difference between the highest MDA level in the Bregalnica River and the lowest level in the Zletovska River was statistically significant (Fig. 1c). This, again, confirmed more intensive influence of pesticide than metal contamination on development of oxidative stress. A marked increase in MDA value was also reported for the gills of *C. carpio* after exposure to the pesticide chlorpyrifos (Yonar et al. 2012), as well as for the gills of goldfish (*Carassius auratus*) after exposure to hexabromobenzene (Feng et al. 2014). Kamunde and MacPhail (2011) even observed a decline in MDA levels in the gills of rainbow trout (*Oncorhynchus mykiss*) after exposure to metal mixture containing Cu, Cd and Zn, claiming that metal mixtures may even induce reductive stress (Kohen and Nyska 2002). They additionally stated that the absence of lipid peroxidation in fish exposed to high levels of Zn can be a result of increased membrane stability and protection provided by this metal, due to its ability to inhibit NADPH-cytochrome c reductase and compete with redox-active metals (Kamunde and MacPhail 2011; Stohs and Bagchi 1994). Hypothetically, this observation can point to an extremely high Zn exposure level in the Zletovska River, especially in the autumn period (1.5 mg L^{-1}; Ramani et al. 2014), as a cause of low MDA level in the gills and intestine of chub from that river. Contrary to oxidative stress, histopathological alterations in the gills and liver of the same chub as used in this study were more pronounced in the metal-contaminated rivers, especially in the Zletovska River, than in the pesticide-contaminated Bregalnica River in both seasons (Barišić et al. 2015; Jordanova et al. 2016).

Comparison of MDA values in chub intestine and gills—autumn of 2012

To compare MDA levels in chub gills and intestine in the autumn period, two-way ANOVA was applied, with river as a second factor (Table 5). If the values obtained at all three rivers were compared together, there was a statistically significant difference between these two organs, with almost twice higher values obtained in the gills (Table 5). If each river was analysed separately, approximately twice higher values were obtained in the gills than in the intestine in both the Bregalnica and Kriva rivers, and the differences were statistically significant. Only in the case of the Zletovska River there was not a statistically significant difference between the two organs, probably indicating that in both organs, the measured MDA values corresponded to basal values (15.9–17.4 nmol g^{-1}). For example, basal MDA levels measured in the gills of the control group of rainbow trout (*O. mykiss*) were even higher and amounted to 27 nmol g^{-1} (Isik and Celik 2008).

Furthermore, in the autumn period, spatial distribution of MDA values in both organs was comparable, with the highest MDA levels obtained in the highly pesticide polluted Bregalnica River. In the Bregalnica River, the herbicide found in the highest concentration was bentazone, a common herbicide in rice cultivation (Stipaničev et al. 2017), previously demonstrated as a potential cause of induction of lipid peroxidation and alteration of activities of antioxidant enzymes in human erythrocytes (Abudayyak et al. 2014). The only difference between the gills and intestine, as already pointed out, was that the response in the gills was twice stronger or, what is perhaps even more important, that majority of fish from pesticide-polluted rivers had increased MDA values in the gills, whereas only some of them had increased MDA values in the intestine. The reason can be probably found in the fact that the gills are continually and directly exposed to polluted river water (Víg and Nemcsók 1989), whereas exposure of the intestine depends on the quantity and type of the food ingested. Our findings are in accordance with previous reports on the gills as the most sensitive tissue to the lipid peroxidation induced by xenobiotics, whose antioxidant potential is weak compared to that of other tissues (Fatima et al. 2000; Sayeed et al. 2003). Therefore, assessment of the oxidative stress in chub from differently contaminated rivers based on MDA response was much more obvious if chub gills rather than intestine were used as a target organ.

Several authors already reported that antioxidant responses vary among different fish tissues. For example, exposure to the organophosphate pesticide diazinon had caused an increase in MDA level in the muscle and kidney of Nile tilapia (*Oreochromis niloticus*), whereas at the same time, it had no effect on the gills and resulted in an MDA decrease in the alimentary tract (Durmaz et al. 2006). Ozcan Oruc et al. (2004)), on the other hand, showed that the gills and kidneys were the organs more affected by oxidative stress than the brain of *O. niloticus* and *C. carpio* after exposure to pesticides, as seen from increased activities of antioxidant enzymes (superoxide dismutase (SOD) in the gills and glutathione *S*-transferase (GST) in the kidneys). It is interesting, however, to observe that at the same time, MDA synthesis was not induced in the gills of those two fish species (Ozcan Oruc et al. 2004), the same as later observed in the rainbow trout (*O. mykiss*) after exposure to the pesticides methyl parathion and diazinon (Isik and Celik 2008). According to Palace et al. (1996), the most responsive indicator of exposure to contaminants that cause oxidative stress is SOD. Lipid peroxidation in the

tissues, on the other hand, is sometimes low or lacking, as a reflection of the protective activity of oxidative enzymes (Ozcan Oruc et al. 2004), which is more or less successful, depending on the fish species. This species-specific response can be further corroborated by the report that the activities of certain biomarkers of oxidative stress were more sensitive to pesticides in *C. carpio* than in *O. niloticus* (Ozcan Oruc et al. 2004), which can be explained by the fact that both oxidative responses and the antioxidant potential of fish differ depending on species habitat and feeding behaviour (Ahmad et al. 2000). The occurrence of observable lipid peroxidation in chub tissues in our study, reflected in increased MDA levels, in contrast to its absence in carp and trout (Ozcan Oruc et al. 2004; Isik and Celik 2008), could be associated with possibly less effective antioxidant defences in the chub *S. vardarensis*, as was previously reported for the chub *Squalius carolitertii* compared to some other fish species, such as barbel (*Luciobarbus bocagei*) and nase (*Pseudochondrostoma* sp.) (Pereira et al. 2013).

Concluding remarks

Research on malondialdehyde concentrations in the organs of Vardar chub (*S. vardarensis*) has pointed out this fish species as highly susceptible to oxidative stress, since, unlike many other fish species, it exhibited a noticeable increase in MDA levels already after exposure to environmentally relevant, sublethal contaminant concentrations. However, significantly increased MDA levels in specimens from the pesticide-polluted Bregalnica River in the autumn period in both studied organs, as opposed to low MDA values in the highly metal-contaminated Zletovska River, indicated higher susceptibility of Vardar chub to development of oxidative stress after exposure to organic compounds than to metals. In addition, between the two chub uptake organs, the gills have been demonstrated as a more sensitive target organ for monitoring of oxidative stress due to their direct and constant contact with contaminants in water, contrary to the intestine, whose exposure depends on the type and quantity of the ingested food. Our research, therefore, has confirmed that MDA increase depends on bioindicator species, target organ, and exposure compounds, which all have to be taken into consideration during assessment of water pollution and consequent oxidative stress of aquatic organisms.

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