Evaluation of multi-biomarker response in fish intestine as an initial indication of anthropogenic impact in the aquatic karst environment

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Abstract

In order to assess the extent of existing anthropogenic influence on biota of the vulnerable karst ecosystem of the Krka River, multi-biomarker approach was applied in the intestinal tissue of brown trout *Salmo trutta* Linnaeus, 1758. Biomarkers of the general stress (total cytosolic proteins), oxidative stress (malondialdehyde), antioxidant capacity (catalase activity, total glutathione) and of an exposure and effect of contaminants, especially metals (metallothionein) and organophosphorous pesticides and metals (acetylcholine esterase activity) were compared in the intestine of fish from the reference site (river source) and downstream of the technological and municipal wastewater impacted site (town of Knin) in two seasons, October 2015 and May 2016. Biological response was additionally evaluated by metal/metalloid concentrations in intestinal cytosol. Site-specific differences were observed as significantly higher As, Ca, Co, Cu, Se and Sr concentrations in intestinal cytosol of fish from the contaminated compared to the reference site in both seasons. Significant seasonal differences existed for Ni, Cd, Mo, Cs and Na, with higher levels in autumn, following the trend of most of the dissolved metal levels in the river water. Impact of improperly treated wastewaters was also confirmed by significantly increased levels of glutathione, total proteins and Foulton condition indices, with 1.5, 1.13 and 1.12 times higher average values in fish from that site compared to the river source, respectively. The other biomarkers showed similar trend and pointed to specific biological changes regarding oxidative stress or metal exposure in fish from the anthropogenically impacted site, especially in autumn, but without significant differences. Thus, the anthropogenic impact still seems to be only moderate, although cytosolic metals and most of the biomarkers in fish intestine were confirmed as initial indicators of pollution impact, which pointed to the need of continuous monitoring of the Krka River in order to protect this natural karst world phenomenon.

Keywords: karst Krka River, brown trout, biomarkers, wastewaters, biomonitoring, cytosolic metals

1. Introduction

Among aquatic environments, karst systems represent the most sensitive areas from both ecological and conservation points of view (Dossi et al., 2007). Specific geological and hydrological constructions contribute to formation of complex ecosystems, which are vulnerable to contaminants due to their ability to be introduced in the karst area through underground and transported rapidly over large distances in the aquifer. In addition, processes of contaminant retardation and attenuation often do not work effectively in karst systems (Brinkmann and Parise, 2012). Although only 10% of the Earth's surface is covered with karst rocks, groundwater from karst aquifers is among the most important drinking water resources for humanity and in this sense, protection of karst hydrologic systems is essential worldwide (Bakalowicz, 2005; Calò and Parise, 2009).

Example of obvious anthropogenic disturbances in the karst systems can be observed in the Krka River, one of the longest rivers in Croatia (72.5 km) situated in karst Dinaric area of the Republic of Croatia. As a result of the constant process of tufa-deposition, the Krka River represents a unique karst phenomenon worldwide and most of its watercourse was proclaimed national park in 1985 (Cukrov et al., 2012). However, only 2 km upstream of the border of the Krka National Park, technological wastewaters of the screw factory and municipal wastewaters of the town of Knin, are released without proper treatment into the river watercourse. Therefore, anthropogenic impact is represented by direct influence of the mentioned pollution sources, as well as indirect influence of agricultural runoff from the surrounding fields, with special emphasis on metals/metalloids contamination from the nearby screw factory and fertilizers (Cukrov et al., 2008; Filipović Marijić et al., 2018). Trace metals are naturally present in the

aquatic ecosystems in very low concentrations, even extremely low in karst rivers (Cukrov et al., 2008). Previous studies already reported low natural metal levels in the river water from the Krka River source, while 2-400 times higher Al, Co, Fe, Li, Mn, Ni, Sr, Ti, and Zn levels were recorded in the technological/municipal wastewaters and the Krka River water under the anthropogenic influence downstream of the town of Knin (Cukrov et al., 2008; Filipović Marijić et al., 2018, Sertić Perić et al., 2018). Wastewater impact was also confirmed by higher densities and diversity of benthic organisms dominated by contamination-tolerant taxa (Sertić Perić et al., 2018). In order to evaluate the extent of anthropogenic influence on the aquatic organisms in the vulnerable karst ecosystem, brown trout (*Salmo trutta* Linnaeus, 1758) was selected as a bioindicator organism, as a typical representative of the Krka River ichthyofauna and moreover, widely spread species in European rivers, which provides the possibility and opportunity for comparison between different regions.

Biological responses to contaminant exposure were for the first time evaluated in the karst area by application of multi-biomarker approach in fish intestine, due to its importance in dietary metal uptake, digestion and nutrient absorption. Most of the biomonitoring studies regarding metal exposure usually involved liver, kidneys and gills as typical indicator organs in fish, while data on the intestinal tissue is still limited, especially regarding fish from the karst rivers. Our previous study involved histological alterations in brown trout intestine and pointed to specific histopathological biomarkers as an indication of pollution impact in the Krka River (Barišić et al., 2018). In the present study we expanded previous findings to demonstrate exposure to and/or effects of environmental contaminants by application of the multi-biomarker approach involving biomarkers of the general stress (total cytosolic proteins, TP), antioxidant defense (catalase activity, CAT and total glutathione, GSH as markers of antioxidant capacities), oxidative stress

(malondialdehyde, MDA as an indicator of oxidative damage), and of an exposure and effect of contaminants (metallothioneins (MT) as biomarkers of metal exposure and acetylcholine esterase activity (AChE) as biomarker of effect on nervous system following exposure to organophosphate and carbamate pesticides, but also other contaminants like metals). The use of multi-biomarker approach is necessary in environments with complex mixtures of contaminants, for the assessment of different biological responses that reflect the environmental quality and for the identification of exposure to contaminants present at low levels in the environment (Monserrat et al., 2007; Cravo et al., 2009).

Biological effects may also link the bioavailability of compounds of interest with their concentrations in target organs and intrinsic toxicity (van der Oost et al., 2003). Therefore, besides biomarker approach, additional biological response was evaluated by the measurement of metal/metalloid concentrations in the intestinal cytosol of brown trout, in order to evaluate metal accumulation in fish since trace elements represent directly introduced contaminants in the Krka River water. After entering the organism, trace metals usually undergo a series of metabolic processes and incorporate into various cellular components. In general, partitioning of metals among subcellular fractions might be grouped in two categories: a) metal-sensitive fractions (MSF): heat-denaturable proteins (HDP), mitochondria and lysosomes and microsomes; b) biologically detoxified metals (BDM): insoluble metal-rich granules (MRG) and heat-stable proteins (HSP) like metallothioneins (MT) and metallothionein-like peptides (MTLP) (Wallace and Luoma, 2003; Urien et al., 2018). HSP fraction was indicated as the most responsive fraction to increased metal exposure (Caron et al., 2018; Urien et al., 2018). In order to evaluate concentrations of metals bound to cytosolic biomolecules, which represent soluble and metabolically available metal fraction (Wallace and Luoma, 2003; Rainbow et al., 2011; Caron et

al., 2018; Urien et al., 2018), our research involved metal and metalloid analyses in the cytosolic fraction of fish intestine.

Hence, our main goals were: 1) to examine the impact of the direct pollution sources (technological and municipal wastewaters) on the karst region and biota using the multi-biomarker approach and cytosolic metals levels as bioindicators in the intestine of brown trout; 2) to evaluate the potential of the intestinal tissue as a novel bioindicator organ in environmental risk assessment due to its importance in food and metal uptake.

2. Materials and methods

2.1. Study area and fish sampling

The study was carried out in the Krka River which is nowadays threatened by the influence of the technological and municipal wastewater inputs. Sampling was performed at two locations, reference (Krka River source) and anthropogenically impacted site, which is situated near the town of Knin and only 2 km upstream of the border of the Krka National Park. This part of the watercourse is the recipient of the technological wastewaters from the screw factory and of municipal wastewaters from the town of Knin (15000 inhabitants), so fish sampling was performed downstream of both outlets (Fig. 1). Previous studies indicated that water ecological status was deteriorated and concentrations of many investigated metals/metalloids were increased at the location under the wastewaters impact compared to the reference site, the Krka River source (Filipović Marijić et al., 2018; Sertić Perić et al., 2018).

Brown trouts (*Salmo trutta* Linnaeus, 1758) were collected in the autumn 2015 (October) (16 specimens from the reference and 20 from the contaminated site) and in the spring 2016 (May) (16 specimens per each site). Fish sampling was performed by electro fishing, according to the Croatian standard HRN EN 14011 (2005). Captured fish were kept alive in an opaque tank with aerated river water until further processing in the laboratory. The biometric data involved measurement of fish total length and body mass, as well as calculation of fish indices: Fulton condition index ($FCI=W/L^3\times100$; Ricker, 1975), hepatosomatic index ($HSI=(LW/W)\times100$; Heidinger and Crawford, 1977) and gonadosomatic index ($GSI=(GW/W)\times100$; Wootton, 1990), where W is the body mass (g), L is the total length (cm), LW is the liver mass (g) and GW is the gonad mass (g). Intestine, liver and gonads were dissected after the fish were anesthetized with tricaine methane sulphonate (MS 222, Sigma Aldrich) in accordance to the Ordinance on the protection of animals used for scientific purposes (NN 55/2013) and then sacrificed. Priborsky et al. (2015) confirmed that exposure of barbell (*Barbus barbus*) to MS 222 for 10 min. does not have a significant impact on haematological profiles, oxidative stress biomarkers and antioxidant enzymes. Accordingly, fish were anaesthetized in groups of 5 in order to shorten the exposure period to less than 10 min., carefully applying the dosage of anaesthetic according to Topić Popović et al. (2012). The whole digestive tract was removed on ice, intestinal part was cut off and cleaned of exterior fat. Afterwards, intestinal fish parasites, acanthocephalans, and the gut content were removed from the intestine and tissue was rinsed with MQ water. Tissues were weighed and then stored in liquid nitrogen until transported to the laboratory, where samples were kept at −80 °C until further analyses.

2.2. Tissue preparation and homogenization

Each sample of intestinal tissue was divided in three parts appropriate for homogenisation procedure related to the GSH measurement, MT measurement and measurement of other biomarkers and metals. Prior to GSH measurement, intestinal tissues were homogenised in five volumes of ice-cold 5% sulfosalicylic acid (SSA) and then centrifuged at 10,000 x g for 10 min at 4 ºC (Biofuge Fresco, Heraeus, Germany). Prior to MT measurement, fish intestinal samples were homogenized in five volumes of 20 mM Tris-HCl buffer, pH 8.6 with 0.5 M sucrose, 0.5 mM phenylmethylsulfonilfluoride (PMSF), 0.006 mM leupeptine, and 0.01% β-mercaptoethanol as a reducing agent. The homogenates were afterwards centrifuged at 50,000 x g for 2 hours at 4 ^{o}C. Samples of fish intestine used for measurement of other biomarkers and metals were homogenised in five volumes of cooled homogenization buffer containing 100 mM Tris-HCl/base (Merck, Germany, pH 8.1 at 4 °C) with 1 mM DTT (Sigma, USA) as a reducing agent, 0.5 mM PMSF (Sigma, USA) and 0.006mM leupeptin (Sigma) as protease inhibitors (Filipović Marijić and Raspor, 2010). In all cases homogenization was performed in an ice cooled tube using Potter-Elvehjem homogenizer (Glas-Col, USA) and the resulting homogenates were afterwards centrifuged in the Avanti J-E centrifuge (Beckman Coulter, USA) at different settings depending on biomarker analyses. For MDA analyses, homogenates were centrifuged at 3,000 x g for 10 min at 4ºC, for analyses of AChE and CAT activity remaining homogenates were centrifuged at 10,000 x g for 30 min at 4ºC to get post-mitochondrial fraction. Lastly, obtained supernatants at 50,000 x g for 2 h at 4ºC represented cytosolic tissue fraction and were used for metal and TP analyses. All obtained supernatants were separated and stored at −80 °C for subsequent analyses.

2.3. Digestion of cytosolic fractions and determination of total dissolved macro and trace elements

Cytosolic fractions were digested in duplicates by addition of oxidation mixture (v/v 1:1), which contained concentrated HNO_3 (Rotipuran® Supra 69%, Carl Roth, Germany) and 30% H_2O_2 (Suprapur®, Merck, Germany) (v/v 3:1). Homogenization buffer was used as a blank and treated the same way as the samples. Digestion was performed in the laboratory dry oven at 85 °C for 3.5 h. Following digestion, samples were diluted with Milli-Q water by dilution factor 20 for Na, K, and Mg, and 5 for the remaining elements. Indium (1 μg L^{-1}, Indium Atomic Spectroscopy Standard Solution, Fluka, Germany) was added to all solutions as an internal standard to correct the changes in peak intensities due to instrumental drift and matrix suppression (Fiket et al., 2007). During the analyses, the validation of acid digestion efficiency of cell cytosolic fraction was performed by the digestion of dogfish muscle certified reference material for trace metals (DORM-2, National Research Council of Canada, NRC, Canada). The recovery means (± SD, n=5) of the trace elements studied from the reference material (As, Cd, Co, Cu, Fe, Mn, Ni, Se, Tl and Zn) are presented in Table 1.

High resolution inductively coupled plasma mass spectrometer (HR ICP-MS, Element 2; Thermo Finnigan, Germany), equipped with an autosampler SC-2 DX FAST (Elemental Scientific, USA) was used to analyze 20 macro and trace elements. Measurements of ^{82}Se, ^{85}Rb, ^{98}Mo, ^{111}Cd, ^{133}Cs, and ^{205}Tl were operated in low resolution mode; of ^{23}Na, ^{24}Mg, ^{42}Ca, ^{47}Ti, ^{51}V, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, and ^{86}Sr in medium resolution mode; and of ^{39}K and ^{75}As in high resolution mode. The external calibration was performed using 2 calibration solutions. For macro elements, multielement stock standard solution containing Ca 2.0 g L^{-1}, Mg 0.4 g L^{-1}, Na 1.0 g L^{-1}, and K 2.0 g L^{-1} (Fluka, Germany) was used for preparation of calibration standards. Calibration solution for the trace elements was prepared by dilution of 100 mg L^{-1} multielement

stock standard solution (Analitika, Czech Republic) supplemented with Rb (Sigma-Aldrich, Germany) and Cs (Fluka, Germany). The accuracy and the precision of HR ICP-MS measurements was tested using quality control sample for macro-elements (QC Minerals, Catalog number 8052, UNEP GEMS, Burlington, Canada) and for trace elements (QC trace metals, catalog no. 8072, UNEP GEMS, Burlington, Canada). A generally good agreement was observed between our data and certified values, with the following recoveries (%) (based on two measurements in control sample for trace elements and two measurements for macro elements): As (100.7 ± 6.7), Ca (95.5 ± 1.6), Cd (95.1 ± 0.7), Co (98.3 ± 0.2), Cu (97.9 ± 0.0), Fe (99.7 ± 2.6), K (92.5 ± 4.2), Mg (93.5 ± 4.9), Mn (98.1 ± 0.0), Na (96.2 ± 3.4), Ni (94.1 ± 5.0), Se (100.8 ± 6.1), Sr (100.8 ± 0.6), Ti (80.3 ± 0.5), Tl (96.0 ± 0.8), V (101.1 ± 0.3), and Zn (96.0 ± 1.3). Limits of detection (LOD) were calculated as three standard deviations of ten consecutive trace element determinations in the blank sample (100 mM Tris-HCl/Base, 1 mM dithiotreitol) digested according to the procedure for cytosols. LOD for macro elements, in µg/g, were as follows: Ca, 1.07; K, 0.112; Mg, 0.024; and Na, 0.320, and LOD for trace elements, in ng/g, were as follows: As, 6.72; Cd, 0.430; Co, 0.266; Cs, 0.102; Cu, 13.5; Fe, 141; Mn, 0.810; Mo, 0.680; Ni, 8.55; Rb, 0.339; Se, 2.93; Sr, 1.09; Ti, 4.76; Tl, 0.001; V, 2.86; and Zn, 635.

2.4. Biomarkers determination

2.4.1. Determination of AChE and CAT activities

The AChE and CAT activities were determined in postmitochondrial fraction (S10). AChE was analysed according to the method described by Ellman et al. (1961). The reaction mixture consisted of the sample, 100 mM Tris-HCl buffer (pH 7.5 at 25 ºC) and 1.6 mM DTNB (5, 5-

dithiobis-2-nitrobenzoic acid). After incubation in dark for 15 min., measurement of the enzyme activity was initiated by the addition of 20 mM acetylthiocholine iodide. The increase in absorbance at 412 nm was monitored immediately following the addition of acetylcholine iodide. The enzymatic activity was expressed as nmol of acetylthio-choline hydrolysed per min per mg of protein, using the absorption coefficient of 13.6 mM^{-1} cm^{-1} for calculations (Stepić et al., 2013).

Measurement of the CAT activity was performed spectrophotometrically at 240 nm and 25 ºC following the method by Claiborne (1985). According to the procedure, sodium phosphate buffer (50 mM, pH 7.0) and hydrogen peroxide (30%) were used to prepare 15.8 mM H_2O_2, which was added to 10 times diluted sample. The specific enzyme activity was expressed as µmol of degraded H_2O_2 per min per mg of protein calculated with a molar extinction coefficient of 43.6 M^{-1} cm^{-1}. Protein concentrations in S10 fractions were determined by the method of Lowry et al. (1951).

2.4.2. Determination of GSH levels

Total GSH concentration was measured in ten-times diluted supernatants using a spectrophotometric DTNB-GSSG reductase recycling assay (Tietze, 1969). The procedure for the microtiter plate assay is adapted from the protocol described by Rahman et al. (2006). All solutions were made in 0.1M potassium phosphate buffer with added 1 mM EDTA disodium salt, pH 7.5. Volume of 150 µL of a solution containing DTNB (3.79 mM) and GR (glutathione reductase; 6 U/mL) was added to the sample in the plate. The plate was mixed and left in dark for 5 minutes. Following, 50 µL of NADPH (0.192 mM) solution was added and the absorbance at

412 nm was measured in intervals of 1 min for 5 min. GSH standards (3.125-25 nM mL^{-1}) were prepared in 0.5% SSA and a calibration curve was used to calculate the GSH levels. The values were expressed as nmol of GSH per g of wet tissue mass.

2.4.3. Determination of the MDA concentration

Determination of MDA concentration was performed spectrophotometrically after the reaction of MDA with 2-thiobarbituric acid (TBA) according to Botsoglou et al. (1994) and Ringwood et al. (2003). Firstly, a 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) was added to sample supernatant. Samples were then vortexed and cooled for 15 min. Next, these mixtures were centrifuged in the Biofuge Fresco centrifuge, (Heraeus, Germany) at 4000×g for 15 min at 4 °C and obtained supernatants were transferred to 1.5-mL Eppendorf® tubes. Following, TBA (Alfa Aesar, Germany) dissolved in Milli-Q water was added. Tubes were then heated for 30 min at 100 ºC producing a pink, fluorescent product. Samples were left to cool and transferred into microplate. The absorbance was set to 535 nm wavelength and values were read at the spectrophotometer/fluorometer Infinite M200 (Tecan, Switzerland). To calculate the MDA values, the calibration curve was constructed using 8 concentrations (2-100 µM) of MDA (Aldrich, USA) which was prior dissolved in 1N HCl (Kemika, Croatia). Homogenization buffer was used as a blank and treated in the same way. Values were obtained as µM and finally calculated as nmol of MDA per gram of wet tissue mass.

2.4.4. Determination of MT concentrations

MT determination involves ethanol/chloroform precipitation steps. Afterwards obtained pellets were washed with 87% ethanol and 1% chloroform in homogenizing buffer, centrifuged at 6000 ×g for 12 min and dried under nitrogen gas stream. Pellets containing MT were dissolved by addition of 35 µL of both 0.25 M NaCl and a solution of 4 mM EDTA/ 1M HCl. The thiol group content was analyzed using DTNB dissolved in 0.2 M Na-phosphate/2 M NaCl, pH 8. The absorbance was read at 412 nm at spectrophotometer/fluorometer (Infinite M200, Tecan, Switzerland). The reduced glutathione (GSH) was used as a reference standard (2.5-30 µg GSH) and obtained calibration curve was used to calculate the values. MT concentrations were expressed per total cytosolic proteins (µg MT mg^{-1} proteins) which were determined by the method of Lowry et al. (1951).

2.4.5. Determination of total cytosolic proteins concentrations

The concentrations of total proteins were measured according to Lowry et al. (1951). Reagent A (copper tartrate, Bio-Rad) and Reagent B (Folin reagent, Bio-Rad) were added to 20 times diluted S50 samples. After 15 min waiting and appearance of a blue color, total proteins were measured on a photometer at 750 nm wavelength (Infinite M200, Tecan, Switzerland). Calibration was accomplished using a bovine serum albumin (BSA) (Serva, Germany) as a reference standard (0.25-2 mg ml^{-1} BSA).

2.5. Statistical analyses

311 Statistical analyses were performed using SigmaPlot 11.0 (Systat Software, USA). Data are
312 presented as mean ± standard deviation (S.D.). Variability of metal concentrations and biomarker
313 values in fish intestine between two seasons and two sites were tested by Mann-Whitney U-test,
314 since assumptions of normality and homogeneity of variance were not always met. Correlation
315 among different parameters was performed using Spearman correlation analysis. Levels of
316 significance of certain statistical test are indicated in the text.
317
318 **3. Results**
319
320 3.1. Fish biometric characteristics
321
322 Average total length and body mass of *S. trutta* specimens from the karst Krka River did
323 not show spatial but pointed to seasonal differences, with significantly higher fish biometric
324 parameters in the autumn season at both locations (Table 2). Gonadosomatic indices followed the
325 same trend, with significantly higher levels in the autumn season at both locations, while HSI and
326 FCI had higher values in spring samples. In addition, FCI of fish from the Krka River
327 downstream of Knin were significantly higher compared to fish from the Krka River source in
328 both seasons (Table 2).
329
330 3.2. Cytosolic metal/metalloid concentrations in fish intestine
331
332 The results on metal/metalloid concentrations in the metabolically available intestinal
333 cytosolic fraction are the first of this kind for *S. trutta.* Descending order of metal/metalloid

levels with concentrations higher than 100 µg kg^{-1} are shown in Fig. 2a (the highest levels of Zn and Fe in intestinal cytosol), while the ones with the concentrations lower than 100 µg kg^{-1} are shown in Fig. 2b (the lowest levels of Cs and V in intestinal cytosol). Concentrations of macroelements in the intestinal cytosol of brown trout were the highest for K and Na, as presented in Fig. 3.

Total cytosolic metal/metalloid concentrations in brown trout intestine were significantly higher in fish from the contaminated compared to the reference site, in both seasons for Co and Se, in autumn for As and Cu, and in spring for Ca and Sr (Fig. 2). Average levels of these metals were 2-4 times higher in fish from the contaminated compared to the reference site. The same trend of higher accumulation in fish from the contaminated site was valid for V, Ti and Zn concentrations, but these differences were not significant in any season. On the other hand, significantly higher metal levels in the intestinal cytosols of fish from the Krka River source compared to the contaminated location were evident for Cd (2-27 times), Cs and Tl (2 to 3 times) in both seasons, and for Ni (6 times) only in autumn season (Fig. 2). Among 20 measured cytosolic metals/metalloid, there were no unique patterns observed for Fe, K, Mg, Mn, Mo, Na and Rb, which levels were mostly comparable or slightly higher either at contaminated or reference site but without any significant differences (Figs. 2, 3).

Few cytosolic intestinal metals/metalloids showed seasonal differences, which were significant for As, Cs, Na and Ni in brown trout from the river source and for Mo and Cd in fish caught downstream of the town of Knin. Each of these elements followed the same trend of significantly higher concentrations observed in autumn than spring campaign (1.2-10 times), with exception of As which levels were higher in spring than autumn for 13 times (Figs. 2, 3).

358

359 3.3. Biomarker responses in brown trout intestine

360

361 3.3.1. Biomarker of exposure to organophosphorous pesticides and metals - AChE

362

363 In the present research, average values of AChE activity in brown trout intestine did not
364 show any spatial or temporal significant differences. However, AChE activity was decreased in
365 fish dwelling at the pollution impacted site and this difference was more pronounced in spring
366 indicating possible pesticide or metal exposure. Average values of AChE activity found in our
367 study ranged from 7.52±1.66 to 9.36±3.49 nmol min^{-1} mg^{-1} prot. if both seasons and both
368 locations were considered (Fig. 4a).

369

370 3.3.2. Biomarkers of antioxidative capacity - CAT and GSH

371

372 There was no unique spatial or seasonal pattern in CAT activity in brown trout intestinal
373 tissue (Fig. 4b). Slightly higher values were observed in fish from the contaminated site
374 compared to the reference location in autumn, while seasonal differences showed higher CAT
375 activity in spring compared to autumn season in fish from the river source. Average values of
376 CAT activity ranged from 13.51±5.64 to 18.34±6.87 μmol H_2O_2 min^{-1} mg^{-1} prot. if both seasons
377 and both locations are considered and there were no significant season- or site-specific
378 differences observed.

379

380 GSH levels in the brown trout intestinal tissue showed both spatial and seasonal significant
381 differences (Fig. 4c). Spatial differences were significant in autumn, when 1.5 times higher GSH

382 levels were recorded in fish from the contaminated site compared to the reference site. Seasonal
383 difference was observed only at the contaminated site with significantly higher values obtained in
384 autumn (mean±S.D.: 1642.3±256.6 nmol g^{-1} w.w.) than in spring (mean±S.D.: 1277.7±289.7
385 nmol g^{-1} w.w.), while levels observed in fish from the Krka source were almost the same in both
386 seasons (Fig. 4c).
387
388 3.3.3. Biomarker of oxidative stress – MDA
389
390 MDA concentrations showed slightly higher average values in fish originating from the
391 location downstream of wastewater discharges, especially in autumn, but still not significantly.
392 Also, seasonal differences were not significant although average MDA concentrations were
393 higher in autumn (152.97±58.36 and 166.1±41.19 nmol g^{-1} w.w. at the reference and
394 anthropogenically impacted site, respectively) than in spring (147.11±36.71 and 148.7±44.63
395 nmol g^{-1} w. w. for the reference and anthropogenically impacted site, respectively) (Fig. 4d).
396
397 3.3.4. Biomarker of metal exposure – MT
398
399 Significantly higher MT levels were evident in the intestinal tissue of fish from the Krka
400 River source in the spring compared to the autumn season. As seen in Fig. 4e, average MT
401 concentrations in spring (7.03±2.07 µg MT mg^{-1} prot.) were almost two times higher than in
402 October (4.26±0.53 µg MT mg^{-1} prot.) in fish from the reference location. Spatial differences
403 were not significant, although higher MT levels were evident in spring in fish from the reference
404 location compared to the contaminated one.
405

406 3.3.5. Biomarker of a general stress - TP

407

408 Site specific differences in protein levels pointed to higher values in fish caught near the
409 town of Knin than the reference location (Fig. 4f). These differences were significant only in
410 autumn, with 1.13 times higher average TP levels in fish from the wastewater impacted site (54.1
411 mg g^{-1} w. w.) than the reference site (47.8 mg g^{-1} w. w.). At both locations, TP levels were a bit
412 higher in spring season than in autumn, but not significantly (Fig. 4f).

413

414 # 4. DISCUSSION

415 4.1. Biometric characteristics

416 Sampled population of brown trout from the karst river confirmed seasonality of biometric
417 parameters in relation to fish physiology. Higher values of total length, body mass and GSI
418 observed in autumn season at both locations are in accordance with brown trout biology and
419 spawning period which occurs in late autumn (Mrakovčić et al., 2006, Hajirezaee et al., 2012).
420 On the other hand, the opposite trend of HSI and FCI is probably a result of the mobilization of
421 energy reserves needed for reproductive development, as well as of higher food supply during the
422 spring period (Moddock and Burton, 1999). In both seasons, significant site specific differences
423 suggested the influence of pollution gradient on FCI, i.e. higher FCI levels downstream of the
424 town of Knin compared to the Krka River source might be associated to higher concentrations
425 and consequently better availability of nutrients at the anthropogenically impacted site (Lambert
426 and Dutil, 1997; Couture and Rajotte, 2003). In the literature data the opposite trend of lower FCI
427 values in metal polluted locations is also frequently observed (Laflamme et al., 2000; Rajotte and

Couture, 2002; Couture and Rajotte, 2003), rising to conclusion that the wastewater impact near the town of Knin did not induce defense mechanism of fish in a way to require a lot of energy which would result in decreased FCI.

4.2. Cytosolic metal/metalloid concentrations in fish intestine

Intestinal metal/metalloid levels in fish cytosol reflect soluble metal fraction which might be bound to cytosolic biomolecules and correspond to the dietary metal uptake route. Previous studies have already reported ecological status and total dissolved metal/metalloid concentrations in the river water from the same locations (Filipović Marijić et al., 2018; Sertić Perić et al., 2018). In these studies, few physico-chemical water parameters (temperature, conductivity, total dissolved solids and total water hardness) indicated slightly degraded ecological conditions at the anthropogenically impacted site and increased dissolved metal levels in water at the same site compared to the river source, especially for Fe, Li, Mn, Mo, Sr, Rb and Ca. The highest increase was recorded for Fe and Mn, which levels were 17 times and 38 times higher near town of Knin compared to the reference site, respectively, while other metals showed the increase in average levels from 1.2 to 2.2 times (Filipović Marijić et al., 2018; Sertić Perić et al., 2018).

However, despite these differences, metal levels along the Krka River watercourse were rather low and mostly comparable to metal levels reported for other karst ecosystems (Cukrov et al., 2008, 2012, Dossi et al., 2007) or lower compared to anthropogenically impacted world rivers (Filipović Marijić et al., 2018). This can be explained by effective self-purification process of the Krka River, which is contributed by the input of underground water, sinking of contaminants in lake sediments and changes in water levels (Cukrov et al., 2008; Filipović Marijić et al., 2018).

Obtained results on environmental conditions in the river water were compared with the metal/metalloid accumulation in fish intestine, which mostly reflected the similar pattern as already recorded for total dissolved metals/metalloids in the river water (Filipović Marijić et al., 2018; Sertić Perić et al., 2018) and therefore, indicated bioavailability and dietary intake of these metals in fish intestinal tissue. Accumulation of metals in fish from the location influenced by technological and municipal wastewaters was significant and over 3 times increased for Co, As and Sr when compared to their levels in fish from the river source (Figs. 2a, b). However, few elements showed the opposite trend with significantly higher concentrations in fish from the Krka River source, like Cd, Cs, Tl. Such results are in accordance with total and cytosolic concentrations of metals/metalloids in the liver of the same *S. trutta* from the reference location (Dragun et al., 2018), while levels of these elements in water were uniform along the Krka River watercourse, meaning that their concentrations in the water of the river source were comparable to those in the polluted area (Filipović Marijić et al., 2018; Sertić Perić et al., 2018). Increased metal levels in fish tissues from the Krka River source might be of natural origin, which is in the case of Cd mobilization of naturally occurring Cd, especially from dolomites in the karst area (Cukrov et al., 2008). Diet content might be an important source of Tl as already observed in juvenile fathead minnows by Lapointe and Couture (2009), although the bioaccumulation of waterborne Tl was shown to be more rapid than dietborne but both exposure routs were suggested as a risk of toxicity. However, the cause of higher metal concentrations in fish from the reference location cannot be definitely explained without further investigations, which should involve metal measurement in fish food and river sediment as their possible sources, especially if considering intestine as an organ of food uptake and its importance in fish digestion and nutrient absorption. Analysis of metals in the gut content of some fish species like European chub (*Squalius cephalus*) (Filipović Marijić and Raspor, 2012), carp (*Cyprinus carpio*) (Kraal et al.,

1995), pike (*Esox lucius*) and bream (*Abramis brama*) (Rajkowska and Protasowicki, 2013) and rainbow trout (*Oncorhynchus mykiss*) (Kamunde et al., 2002) have already showed the importance of dietborne metal intake.

Observed seasonal differences in cytosolic intestinal metal levels might be linked to fish physiology. Most metals/metalloids showed higher levels in autumn than in spring, probably as a result of fish physiological changes related to the reproductive period of brown trout in late autumn. Dependence of metal levels upon fish reproductive period can be explained by the fact that essential metals have important roles in fish metabolism, as constitutive part of proteins and other important biological molecules (Miramand et al., 1991, Filipović Marijić and Raspor., 2010, 2014). In addition, Sertić Perić et al. (2018) reported that concentrations of total dissolved metal levels in the river water were also higher in autumn than spring period.

Comparison of metal/metalloid concentrations in cytosolic fraction of brown trout intestine (Figs. 2, 3) with other literature data was possible only for cytosolic metal levels in intestine of European chub from the Sava River, which showed the same descending order of metal levels and even comparable concentrations for Zn>Fe>Cu>Mn>Cd (Filipović Marijić and Raspor, 2012).

4.3. Biomarker responses in brown trout intestine

Combined use of set of different biomarkers enables a more comprehensive and integrative assessment of environmental quality (Broeg and Lehtonen, 2006; Humphrey et al., 2007). In the

498 present study multi-biomarker approach was applied, in order to assess biological responses of
499 native fish exposed to the mixture of contaminants in the karst aquatic environment.
500
501 Inhibition of AChE activity is commonly used as a biomarker of organophosphorous and
502 carbamate exposure in both aquatic and terrestrial environments (Lionetto et al., 2011). However,
503 inhibition of AChE activity might be caused by other contaminants such as heavy metals,
504 polycylic aromatic hydrocarbons or detergents (Elumalai et al., 2007; Richetti et al., 2011), which
505 also might play important role in AChE activity in the present study. Our results on decreased
506 AChE activity, although not significantly, in fish intestine from the area near the town of Knin in
507 both seasons (Fig. 4a), might indicate metal and fertilizer influence on AChE activity inhibition
508 in fish caught downstream of the polluted area. Although this decrease was not significant in the
509 fish intestine (Fig. 4a), it was discernible especially in spring, as period of crop germination and
510 increased usage of fertilizers. In addition, many cytosolic metals in fish intestine had higher
511 levels at the contaminated site than at the reference site (Fig. 2, 3), possibly affecting enzyme
512 inhibition as well. Correlation analysis confirmed significantly negative correlation of AChE with
513 Zn ($r = - 0.59$, $p<0.05$), Fe ($r = - 0.63$, $p<0.01$), Mn ($r = - 0.70$, $p<0.01$) and Sr ($r = - 0.89$,
514 $p<0.001$) levels in intestinal cytosol, indicating metal influence on AChE inhibition. Szabo et al.
515 (1991) reported that intestine of rainbow trout was an organ with the lowest AChE activity in
516 comparison to the brain, muscle and heart. In the same research, trout was described as a species
517 with the lowest AChE activity in the intestinal tissue in comparison to 11 other fish species,
518 which average value was 10 nmol min^{-1} mg^{-1} prot. In the present research, average values of
519 AChE activity in fish from the reference site were around 9 nmol min^{-1} mg^{-1} prot. (Fig. 4a) which
520 is in agreement with the mentioned literature values.
521

Pollution impact near the town of Knin was also evaluated by two biomarkers of the antioxidant capacities, CAT and GSH. Our results suggest that fish were subjected to oxidative stress according to slightly higher CAT activities (Fig. 4b), as well as by the significantly higher values of GSH in autumn at contaminated compared to the reference site, respectively (Fig. 4c). CAT activity has already been measured *in vitro* and *in vivo* in the intestine of freshwater fish *Oreochromis niloticus* (Atli et al., 2006) and the values in control group (161.7±15.3 μmol H_2O_2 min^{-1} mg^{-1} prot.) and in fish exposed to Ag, Cd, Cr, Cu and Zn (mostly ranging from 25 to 225 μmol H_2O_2 min^{-1} mg^{-1} prot. depending on the metals and their concentrations) were higher compared to brown trout from the karst Krka River (ranging from 13.51 to 18.34 μmol H_2O_2 min^{-1} mg^{-1} prot., Fig. 4b). In our study, significant correlation between CAT activity and cytosolic metal levels was confirmed for Mo ($r = 0.71$, $p<0.01$) and Co ($r = 0.88$, $p<0.05$) in the intestine of fish from the location downstream of the wastewater outlets.

GSH is involved in different metabolic and transport processes, the protection of cells against toxic effects of different compounds, including oxygen reactive species and heavy metals (Meister and Anderson, 1983; Canesi et al., 1999). In fish from the Krka River, the significant increase in GSH levels was 1.5 times in fish from the contaminated compared to the reference site in autumn and also 1.16 times in spring but without significant difference (Fig. 4c). GSH showed significant seasonal differences, with significantly higher levels in autumn than spring in fish from the wastewater impacted site (Fig. 3c). Such results are in accordance with the higher metal concentrations in intestinal cytosol of fish from the contaminated site in autumn, and therefore suggested possible impact of metals on oxidative stress (Fig. 2, 3). There are many literature data confirming that metals affect the cell antioxidant system efficiency, like for example Cu, Se and Mo. Liu et al. (2005) reported that longer exposure to different concentration

546 of Cu induced a significant increase of GSH content in liver of freshwater fish *Carassius auratus*.
547 Study on fish *Piaractus mesopotamicus* showed that Se supplementation helped lessen free
548 radical damage and boosts immune system function (Biller-Takahashi et al., 2015). GSH levels
549 and CAT activity at the contaminated site were also in accordance with Mo concentrations
550 pattern, which could be due to the formation of molybdate oxoanion which is known to cause the
551 increase in the activities of antioxidant enzymes like super oxide dismutase (SOD), glutathione
552 peroxidase (GPOX) and catalase (CAT) (Panneerselvam and Govindasamy, 2004). To our
553 knowledge, there is no literature data on GSH levels in the fish intestinal tissue and our results
554 can only indicate that GSH levels in the intestine of brown trout were in range of the values
555 observed by Otto and Moon (1996) in the liver (1539±238 nmol g^{-1} w. w.) and kidney (1993±66
556 nmol g^{-1} w. w.) of the adult rainbow trouts.
557
558 The elevated concentration of MDA directly reflects oxidative stress in the organism as a
559 consequence of lipid damage caused by free radicals (Banerjee et al., 1999; Dragun et al., 2017).
560 In our study intestinal MDA levels did not show significant site- or season-specific differences.
561 Slightly higher MDA concentrations were only observed in fish caught near the town of Knin in
562 autumn (Fig. 4d). Such results are in accordance with CAT and GSH results which pointed to
563 moderate evidence of oxidative stress, therefore oxidative stress damages by means of MDA
564 production were not observed. Metal catalyzed formation of reactive ROS capable of damaging
565 tissues such as DNA, proteins and lipids has already been documented. For example, significant
566 effect of dietary Fe on MDA levels in the intestine and liver of rainbow trouts was observed,
567 which was reflected as small but persistent elevation of intestinal MDA values positively
568 correlated with increasing Fe levels in the gut (Carriquiriborde et al., 2004). On the other hand, a
569 research on dietary Cu and Cd in Atlantic salmon revealed that no significant increase in tissue

570 MDA levels was observed in the intestine of fish exposed to dietary Cd, while dietary Cu had a
571 direct effect on lipid peroxidation even at relatively low concentrations (Berntssen et al., 2000).
572 Greani et al. (2017) investigated the effect of chronic arsenic exposure under environmental
573 conditions on oxidative stress in wild trout and significant increase of MDA levels was observed
574 in muscles, kidney, liver and fins of exposed trouts. In our study, levels of As in intestinal cytosol
575 of brown trouts were higher at contaminated site than at the reference site in both seasons, even
576 significantly in autumn (Fig. 2b), while correlation analysis confirmed significantly positive
577 relation of MDA and Fe ($r = 0.70$, $p<0.01$) and Ni ($r = 0.71$, $p<0.01$) in fish from the
578 contaminated location. However, MDA levels were not significantly higher near the town of
579 Knin compared to the river spring, so the existing contamination in investigated area was not
580 high enough to induce sufficient oxidative damage in fish and was probably counteracted by
581 antioxidant defense mechanisms (CAT, GSH).
582
583 Spatial differences were also observed for TP levels, with significantly higher values
584 recorded in fish caught near the town of Knin in autumn, but only slightly higher levels in spring
585 (Fig. 4f), following the trend of biomarkers of antioxidant capacities and pointing to more
586 stressful conditions for brown trouts at the site under the wastewater impact. Additionally,
587 significantly positive correlation between TP levels and metal levels was observed for Mg ($r =$
588 0.50, $p<0.01$), Cu ($r = 0.69$, $p<0.05$), Mn ($r = 0.74$, $p<0.05$) and Zn ($r = 0.69$, $p<0.05$) in fish from
589 the contaminated site. However, temperature, oxygen levels and salinity are also known as
590 important factors influencing the protein turnover rates in active tissues, but protein synthesis can
591 also be correlated to feeding habits (Peragón et al., 1994). Thus, higher protein content observed
592 in spring at both locations might also suggest that there were more available food sources in

593 spring, especially near the town of Knin, which would be in accordance with the higher FCI and
594 fish masses from that site (Table 2).
595
596 The opposite response compared to other biomarkers was obtained only for MT, which
597 showed higher levels in fish from the reference than polluted location in spring, but without
598 significant differences (Fig. 4e). Metallothionein induction has been widely considered as
599 efficient biomarker for metal pollution in a variety of animal species (Ivanković et al., 2005;
600 Mosleh et al., 2006; Filipović Marijić and Raspor, 2010; Calisi et al., 2013). As one of the main
601 MT roles is the regulation of essential metals like Zn and Cu, and detoxification of nonessential
602 metals like Cd, Hg and Ag, some of these metals might contribute to the higher MT values in
603 brown trout intestine in spring at both sites. At the Krka River source, concentrations of Cd, Cu,
604 and Zn were higher in the spring campaign, although without significant differences, possibly
605 affecting higher levels of MT at this site in spring. MT induction in the intestine of different fish
606 species has already been confirmed by Handy et al. (1999) and Berntssen et al. (1999) after
607 dietary Cu exposure, by Ptashynski and Klaverkamp (2002) after Ni exposure and by Berntssen
608 et al. (2001), Chowdhury et al. (2005) and Roesijadi et al. (2009) after dietary Cd uptake.
609 However, in polluted environment fish are exposed to a mixture of different metals, and even
610 when MT induction is shown, it is generally impossible to connect this elevated synthesis to
611 specific elements. In addition, MT levels may also be affected by other parameters such as
612 season, temperature, size, fish gender or nutritional status (Hylland et al., 1998; Filipović Marijić
613 and Raspor, 2010). Therefore, higher FCI, as well as higher protein content, in the spring
614 campaign at both sites, indicated the enhanced feeding during that period which also might cause
615 higher MT concentrations, which increase was even significant at the reference location (Fig. 3e).
616

5. Conclusions

Biological responses in the intestinal tissue of *S. trutta* from two sites of the karst Krka River in Croatia revealed that anthropogenic impact downstream of the technological and municipal wastewater impact was evident for biomarker of antioxidant capacities (GSH) and general stress (TP) and for numerous metals/metalloid measured in cytosolic intestinal fraction. Concentrations of As, Ca, Co, Cu, Se and Sr were significantly higher at the contaminated site near the town of Knin compared to the reference location and pointed to a rising need of strict monitoring of water quality and health of aquatic organisms in the Krka River. Cadmium, cesium and thallium levels were elevated in the intestinal cytosol of fish from the Krka River source, but further investigation on metal levels in food sources and sediment is needed to explain such pattern. Therefore, intestinal tissue was shown as a useful indicator organ which may reflect metal uptake and biological responses to contaminant effect or exposure caused by dietary pathways from food sources.

Significant biomarker responses in fish intestine, reflected as higher GSH and TP levels, revealed that fish from the polluted area experienced oxidative and general stress. But comprehensive evaluation of the multi-biomarker response, also involving CAT, MDA, AChE and MT, suggested that in fish living downstream from the wastewaters outlets no significant indication of oxidative damage occurred, neither significant correlation with most cytosolic metals/metalloids. Hence, the impact of contaminants on the Krka River still seems to be only moderate but it is of growing concern that both metals and some biomarkers indicated anthropogenic impact on water and organisms near the town of Knin. Thus, with the time, without the proper and continuous monitoring and protection plan of the region, the

consequences might be more ruinous for the whole biota of the Krka River and the national park itself.

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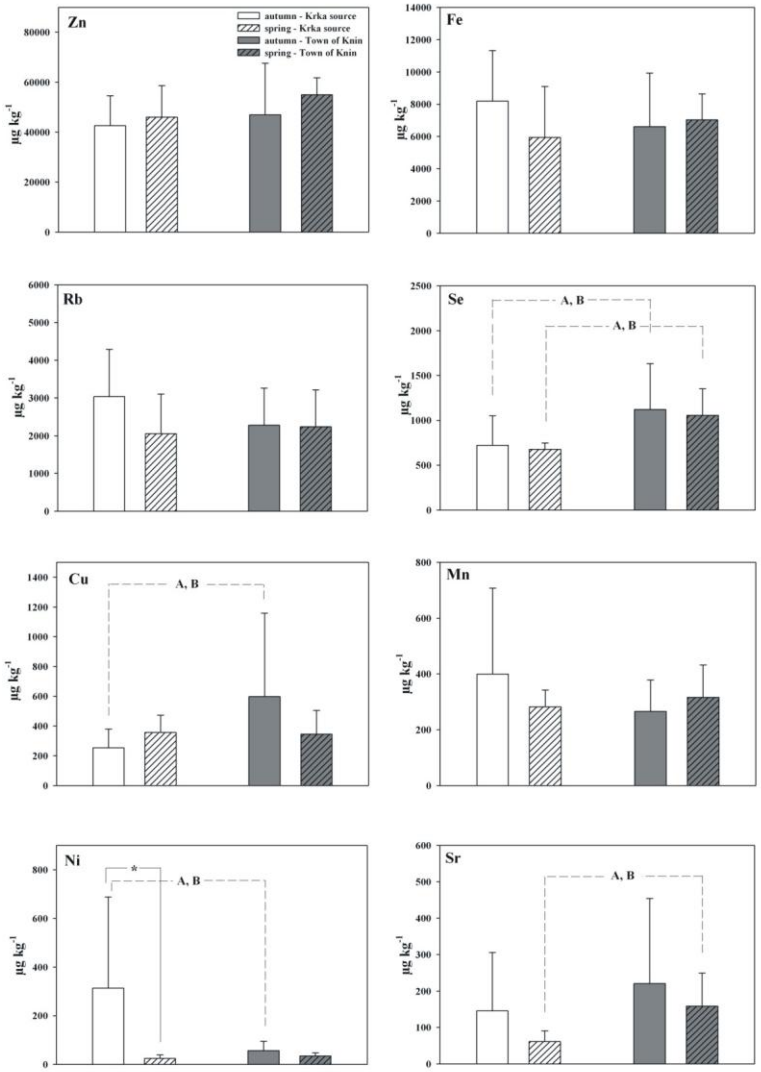
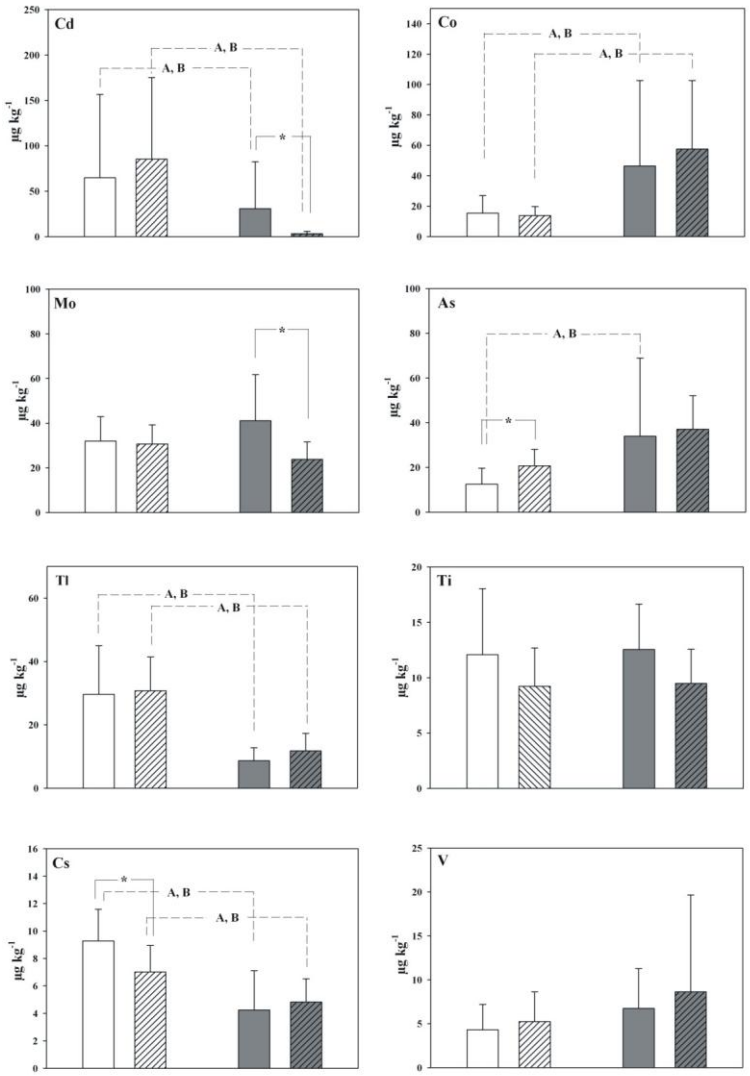
Figure captions:

Figure 1. Sampling locations of the brown trout in the Krka River: 1 reference location - Krka River source; 2 anthropologically impacted location downstream of the technologycal wastewater input from the screw factory (2a) and municipal wastewater outlet from the town of Knin (2b).

Figure 2. Cytosolic trace metals concentrations (mean±S.D., μg kg^{-1}) in intestinal tissue of *S. trutta* from the Krka River at two sampling sites (reference site: Krka River source; contaminated

site: Krka downstream of Knin) in two sampling campaigns (autumn- October 2015 and spring-May 2016); a) elements with average concentrations above 100 μg kg^{-1}, b) elements with average concentrations below 100 μg kg^{-1}. Statistically significant differences (t-test) at $p<0.05$ level between two seasons at each sampling site are marked with asterisk (*) and solid line, and between two sampling sites within the same season are assigned with different superscript letters (A and B) and dashed line. Site legend: white – Krka River source, autumn season; dashed-white – Krka River source, spring season; grey - Krka downstream of Knin, autumn season; dashed-grey - Krka downstream of Knin, spring season.





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941 **Figure 3.** Cytosolic macroelements concentrations (mean±S.D., mg kg^{-1}) in intestinal tissue of *S.*
942 *trutta* from the Krka River at two sampling sites (reference site: Krka River source; contaminated
943 site: Krka downstream of Knin) in two sampling campaigns (autumn- October 2015 and spring-
944 May 2016). Statistically significant differences (t-test) at $p<0.05$ level between two seasons at
945 each sampling site are marked with asterisk (*) and solid line, and between two sampling sites
946 within the same season are assigned with different superscript letters (A and B) and dashed line.

Site legend: white – Krka River source, autumn season; dashed-white – Krka River source, spring season; grey - Krka downstream of Knin, autumn season; dashed-grey - Krka downstream of Knin, spring season.

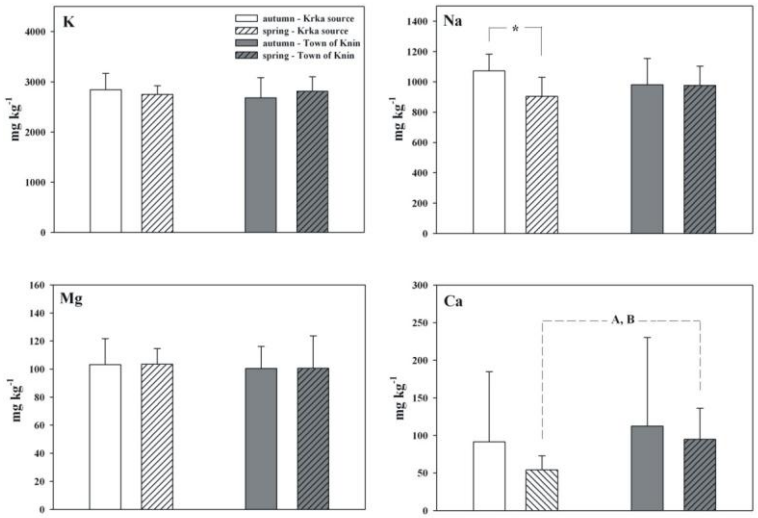
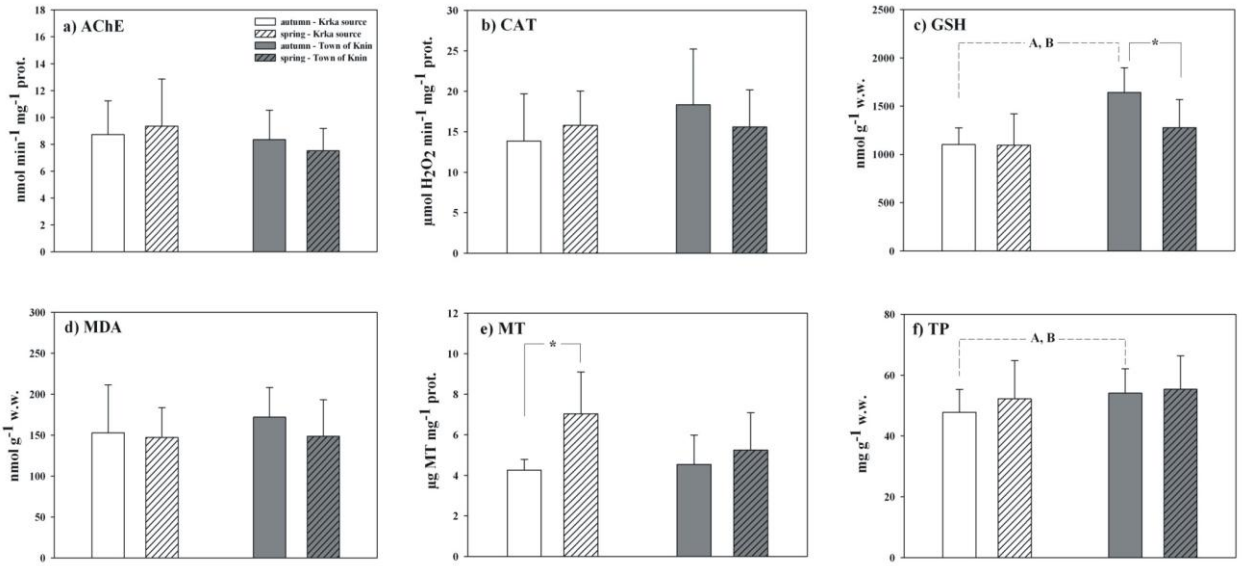


Figure 4. Biomarker levels in intestinal tissue of *S. trutta* from the Krka River at two sampling sites presented as mean values ± S.D. (reference site: Krka River source; contaminated site: Krka downstream of the town of Knin) in two sampling campaigns (autumn- October 2015 and spring- May 2016) (n = 16, except at Krka downstream of the town of Knin in autumn where n = 20). Statistically significant differences (t-test) at $p<0.05$ level between two seasons at each sampling site are marked with asterisk (*) and solid line, and between two sampling sites within the same season are assigned with different superscript letters (A and B) and dashed line. Site legend: white – Krka River source, autumn season; dashed-white – Krka River source, spring season; grey - Krka downstream of Knin, autumn season; dashed-grey - Krka downstream of Knin, spring season.



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963 **Table 1.** Accuracy of HR ICP-MS measurements presented as certified and measured metal values (mean
964 ± S.D., n = 5) in certified reference material (DORM-2, National Research Council, Canada) and
965 calculated recoveries.

Metal	Certified value (DORM-2)	Measured value (DORM-2)	Recovery (%)
		mg kg^{-1}	
As	18.0±1.7	18.6±1.4	103
Cd	0.043±0.008	0.044±0.003	105
Co	0.182±0.031	0.18±0.012	99
Cu	2.34±0.16	2.35±0.073	100
Fe	142±10	142.9±7.13	101
Mn	3.66±0.34	3.71±0.23	101
Ni	19.4±3.1	19.11±1.11	99
Se	1.4±0.09	1.43±0.07	102
Tl	0.004	0.004±0.0005	100
Zn	25.6±2.3	24.28±2.08	95

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971 **Table 2.** Biometric parameters (mean ± S.D. (min.-max.)) of *S. trutta* caught in the Krka River at the
972 reference (Krka River source) and contaminated site (Krka downstream of Knin) in two sampling
973 campaigns (autumn- October 2015 and spring- May 2016). Statistically significant differences (t-test) at
974 $p<0.05$ level between two seasons at each sampling site are marked with asterisk (*) and between two
975 sampling sites within the same season are assigned with different superscript letters (A and B).

	Krka River source		**Krka downstream of Knin**	
	Autumn 2015 n = 16	**Spring 2016** n = 16	**Autumn 2015** n = 20	**Spring 2016** n = 16
Total length (cm)	24.15±4.29***** (18-30.8)	18.36±1.94* (15.2-22.1)	23.16±5.49* (13-31.8)	19.64±3.19* (13.8-26.7)
Body mass (g)	152.71±78.64***** (59.53-303.7)	66.09±19.64***** (36.6-107.2)	165.45±108.96***** (22.15-424.3)	96.01±45.49***** (31.45-200.7)
FCI (g cm^{-3}*100)	1.00±0.08^{A} (0.84-1.13)	1.04±0.06^{A} (0.94-1.15)	1.12±0.10*****$^{, B}$ (0.98-1.38)	1.19±0.09*****$^{, B}$ (1.05-1.37)
HSI (%)	0.92±0.25***** (0.53-1.36)	1.27±0.30***** (0.88-1.97)	0.97±0.12***** (0.76-1.21)	1.50±0.47***** (1.05-3.04)
GSI (%)	3.72±2.49***** (0.11-8.07)	0.40±0.33*****$^{, A}$ (0.13-1.40)	2.30±2.61***** (0.02-7.05)	0.15±0.06*****$^{, B}$ (0.07-0.25)
Sex (M/F/ND)	10/5/1	10/6/0	10/10/0	8/8/0

976 HSI –hepatosomatic index; GSI –gonadosomatic index; FCI – Fulton condition index, ND – not determined

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