



Native Prussian carp (*Carassius gibelio*) health status, biochemical and histological responses to treated wastewaters[☆]

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ABSTRACT

The aim of this study was to assess the impact of treated wastewaters on native wild Prussian carp inhabiting effluent-receiving waters (ERC) receiving municipal and sugar plant treated wastewaters, further downstream waters (DW), and a detached canal unaffected by the WWTP activities. To that end, general fish health status was determined, including plasma biochemical, haematological, oxidative stress and tissue histopathological indices, over three seasons. The greatest tissue alterations were in fall in ERC during sugar beet processing, as hypertrophy of gill epithelial and interlamellar cells, necrosis and lymphocytic infiltration, hyperplasia and hypertrophy of renal tubules, distention of hepatic sinusoids. In fall the lowest leukocytes, lymphocytes and granulocytes (2467 ± 565 , 1333 ± 264 , 1133 ± 488 cells/ μ L respectively), as well as highest plasma ALP (52.7 ± 19.39 U/L) were measured. ERC in fall had the highest ammonium (20 mg/L), nitrite (1.48 mg/L), nitrate (13.4 mg/L), and lowest dissolved O₂ (1.23 mg/L). Gill, kidney and liver alterations, and the highest plasma cholesterol (9.1 ± 1.98 mmol/L) were noted in DW fish in fall. Tissue morphology during sugar cane processing seems a consequence of cellular and structural tissue integrity loss. Structural heterogeneity of gills and spleen was enhanced with increasing concentrations of heavy metals and correlated with oxidative stress ($SOD\ 392.5 \pm 77.28$ U/L). Monogenean infestation was moderate in ERC fish in all seasons compared with DW fish. Prussian carp biological responses to multiple stressors, measured by the effects of WWTP on blood and tissue parameters, reached far downstream and were not of localized nature. This study demonstrated that in aquatic environments impacted with complex contaminants acting synergistically, causal relationships between biological responses and environmental stressors should be interpreted. Integrated histopathological, haematological and biochemical findings are valuable biomarkers for native fish adaptive patterns and monitoring of water quality/pollution of freshwater ecosystems.

Fish biological responses to multiple stressors of treated wastewaters were pronounced in specimens captured from both effluent and downstream waters.

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1. Introduction

Many complex freshwater pollutants may be found in treated wastewaters and exert their effects on aquatic ecosystems and aquatic biota. Fish rapidly accumulate some toxic substances and respond to low concentrations of environmental pollutants (Radić et al., 2013). Fish are thus frequently used model systems in assessing these effects as they impact important physiological functions and thereby the overall health status. The biological effects may affect all levels of biological organization, and it is thus a challenge to identify adequate

biomarker indicators of toxic effects, especially since the treated wastewater effluents are chemically complex, constantly changing and biologically active. The inclusion of biomarkers as functional measures of exposure to stressors in field surveys of contaminated sites is increasingly being reported (Bernet et al., 2000, 2001; Owen et al., 2008; Kaur and Dua, 2014). It has been suggested that examination of haematological indices, plasma chemistry and oxidative stress could serve as a valuable diagnostic tool in assessing fish health impairment (Stoskopf, 1993), while their aberrations from standard values might be reflected in the histopathological tissue lesions (Bernet et al., 2001).

Prussian carp (*Carassius gibelio*) is widely distributed in Europe and Asia and commonly stocked together with carp (*Cyprinus carpio*). It inhabits still water bodies and lowland rivers, can tolerate pollution and low oxygen concentrations. It invaded European ponds,

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eutrophic lakes, canals, and small water reservoirs (USFWS, 2012). Prussian carp grows and reproduces rapidly, and competes with native fish for food and space. Juveniles frequently occur in complex habitats like reed belts. Presently it is the most dominant fish species in lentic and slowly running aquatic habitats (Lusk et al., 2010). Cyprinid fish, particularly carp, have often been included in studies where they were cage held/exposed *in situ* in municipal wastewater treatment plant (WWTP) effluents or in rivers receiving municipal WWTP effluents, while less is known of the biological effects on native wild carp captured from riverine sites influenced by the WWTP treated wastewater (Snyder et al., 2004; Huang et al., 2007; Barber et al., 2011; Karadag et al., 2014). In cage exposure studies, fish are held in conditions with restrained movement possibilities, they are often unable to feed adequately for their species requirements, and are generally unable to exert their characteristic behavioral patterns, and thus frequently die of stressful holding conditions (Snyder et al., 2004). Water management activities alter environmental characteristics of water bodies, often creating environmental traps for wild fish, which increase the risk for fish health (Hale et al., 2015). Although wild fish avoid deleterious polluted conditions and move to more favorable locations in order to enhance their survival and well being (Larrick et al., 1978), assessment of biological responses of wild-caught fish in WWTP-related waters will more likely indicate actual ecological effects of treated wastewater. It is of increasing importance to define appropriate indicators of anthropogenic disturbances for monitoring purposes. For example, Index of Biotic Integrity (IBI) has been extensively used in freshwater ecosystems (Jameson et al., 2001; Adams, 2002), and one of major IBI components in freshwater fish is the health status of fish populations, evaluated through presence and abundance of lesions, tumors and other apparently pathological conditions on fish (Adams and Greeley, 1999). In addition to macroscopic lesions, fish health status is also assessed using simple and rapid techniques such as evaluation of blood smears and wet mounts (Ainsworth et al., 1991; Groff and Zinkl, 1999). Examination of blood cells (erythrocytes, leukocytes and thrombocytes) has been widely accepted as an irreplaceable tool for clinicians and researchers in many areas (Densen and Mandell, 1990). Fish leukocytes have been extensively used to investigate immune responses and effects of environmental stress, toxicant exposure, and diseases on individual fish, as well as fish populations and communities (Sniezsko, 1974; Stoskopf, 1993; Zelikoff et al., 2000; Palić et al., 2011).

The aim of this study was to assess the impact of treated wastewaters on native wild Prussian carp inhabiting effluent-receiving waters (receiving municipal and sugar plant treated wastewaters), further downstream waters (County canal), and a detached canal unaffected by the WWTP activities. To that end, general fish health status was determined, and an array of selected plasma biochemical, haematological, oxidative stress and tissue histopathological indices was evaluated. Our hypothesis was that fish captured in both effluent and downstream waters (County canal) would have pronounced biological responses, in relation to season and/or activity of the sugar plant.

2. Materials and methods

2.1. WWTP and the sampling sites

The study was carried out in spring, summer and fall of 2014. The samplings of water and fish were conducted throughout the treatment process of a Croatian municipal WWTP serving a city of Virovitica, also receiving hospital and sugar plant wastewaters. The sugar plant was active in spring and fall (processing pre-washed sugar cane and sugar beet, respectively), and inactive in summer. Sugar plant con-

tributed with 47,925 m³ (15.94%) in spring and 75,000 m³ (30.83%) in fall. Hospital contributed with 4083 m³ (1.36%) in spring, 3977 m³ (2.65%) in summer, and 4286 m³ (1.76%) in fall. Daily total average of treated wastewater was 6880 m³ in spring, 4850 m³ in summer, and 8109 m³ in fall.

WWTP treatment includes primary and secondary processes, including settling tanks, grit chambers, activated sludge biological process, aeration tanks, secondary tanks for removing the biomass and other suspended particles. The resultant final treated effluent is discharged into a natural water canal (site no. 2 in further text). This canal further downstream receives additional communal treated water from a biological treatment plant serving a small suburb, widens to enter a County canal (site no. 3 in further text) which eventually discharges in the river Drava.

Thus, water and fish from three locations were sampled: (1) stream unaffected with the WWTP activities, although not pristine (45°49'33"N–17°22'28"E), (2) WWTP effluent-receiving canal (45°54'3"N–17°29'6"E), (3) County canal further downstream, approximately 12 km from the point of discharge of treated water (45°51'57"N–17°33'21"E). The County canal is not under regular monitoring for physico-chemical water parameters. It is of interest for the assessment of fish biological responses in a downstream location from the treatment plant. The three sampling sites were chosen as the best representatives of their capacity as unaffected, heavily impacted, and possibly impacted by the treated wastewaters.

2.2. Fish and tissue processing

Fish were caught by nets and angling; they were manipulated by the competent authorized persons (licensed veterinarians) in accordance with the provisions of national legislation and the Institute's Bioethical Committee (No. BEP-274/2-2012).

In spring, 24 Prussian carp (*Carassius gibelio*) (mean weight 498.80 ± 232.04 g, mean length 213.46 ± 66.94 mm) were captured and examined; in summer 19 Prussian carp (mean weight 112.94 ± 65.64 g, mean length 173.78 ± 30.35 mm); while in fall 45 Prussian carp (mean weight 127.80 ± 97.32 g, mean length 170.22 ± 45.32 mm) were examined. Specimens were randomly sampled, transported live to the laboratory and within few hours sacrificed by overdose of tricaine methane-sulfonate (MS-222, Sigma, St. Louis, Missouri, USA).

Wet mounts of gill filaments and skin scrapings were examined under the light microscope. Blood withdrawal and necropsy were performed immediately and tissues (gills, anterior kidney, spleen, liver) were fixed in 4% neutral buffered formalin, dehydrated through a graded ethanol-xylene series and embedded in paraplast. Sagittal and transverse sections (3–5 µm) were stained with hematoxylin/eosin (H&E) and periodic-acid Schiff (PAS) method. Microphotographs were taken with a digital camera DP 70 Olympus® connected to an Olympus® BX51 binocular microscope, and transferred to Microsoft® AnalySIS Soft Imaging System for interpretation. The presence of histopathological alterations was interpreted qualitatively, and the degree of tissue alteration was determined as slight, moderate and severe, based on severity of the lesions.

2.3. Physico-chemical properties and concentrations of heavy metals in water

Physico-chemical properties of water were analyzed according to the international standards as follows: determination of electrical conductivity, pH, suspended solids, dissolved oxygen, permanganate index, chemical oxygen demand (COD), biochemical oxygen demand after n days (BOD_n), dilution and seeding with allylthiourea, ammo-

nium and phosphorus with spectrometric method, nitrite, total nitrogen by persulfate digestion method, total phosphorus by ascorbic acid method, nitrate by colorimetry, and cadmium reduction (ISO 7888:1985, ISO 10523:2008, ISO 872:2005, ISO 5813:1983, ISO 8467:1993, ISO 15705:2002, ISO 5815:1989, ISO 7150-1:1984, ISO 6878:2004, ISO 6777:1984, SM 4500-NO₃-E, respectively).

Total metal concentrations of heavy metals (Cd, Cr, Pb, Hg, Ni, Zn, Cu, Fe) were analyzed according to the international standards as follows: determination of total cadmium, chromium, and lead by atomic absorption spectrometry-graphite furnace technique (ISO 15586:2003, ISO 15586:2004, ISO 15586:2006); determination of mercury by atomic absorption spectrometry (ISO 12846:2012); determination of nickel, zinc, and copper by inductively coupled plasma optical emission spectrometry (ISO 11885:2007, ISO 11885:2011, ISO 11885:2012); determination of iron by spectrometric method using 1,10-phenanthroline (ISO 6332:1988).

2.4. Haematology, blood biochemistry, oxidative stress

Blood samples were collected from all sampled fish by caudal vein puncture, immediately after their capture. Long syringes were used for blood withdrawal from the caudal artery and vein. Blood smears were immediately prepared in triplicate and air dried. Blood was collected in tubes coated with anticoagulant lithium heparin, centrifuged at 12,000g for 90 s and resultant plasma was frozen at -80 °C until analysis two weeks later.

Blood smears were stained with Diff-quick (Sigma-Aldrich) according to manufacturer's instructions. Leukocyte morphology was examined using bright field microscopy (magnification of 400 X and 1000 X), and differential count performed on minimum of two hundred cells (neutrophils, lymphocytes, monocytes, not including platelets) in at least 10 visual fields per slide at 400 X magnification. The cell morphology was confirmed by examination at 1000 X under oil. The numbers of counted leukocytes were calculated according to Fudge (2000) in order to obtain the standardized total leukocyte count per μ L of blood and their ratio (as %).

Heparinized microhaematocrit capillaries were filled with blood, sealed with clay and centrifuged for 120 s at 12,000g within 20 min from collection. The percentage of packed cells to total volume was determined by direct measurement on StatSpin microhaematocrit capillary tube reader. Haematocrit (Hct) was determined as packed cells volume by the microhaematocrit method (Wedemeyer and Yasutake, 1977).

In blood plasma, concentrations of glucose (GLU), urea (URE), creatinine (CRE), cholesterol (CHOL), triglyceride (TRIG), total proteins (TP), albumin (ALB) and activity of alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT) were determined by Beckman Coulter commercial kits (Olympus Life and Material Science Europe, Ireland) on the Olympus AU 640 biochemistry analyzer (Olympus, Japan).

The activity of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were determined by Randox commercial kits on the Olympus AU 640 biochemistry analyzer. Paraonase activity (PON 1) in serum was assayed by modified method of hydrolysis of paraoxon described by Charlton-Menys et al. (2006) on the Olympus AU 640. Enzyme activity was presented in U/L (1 μ mol *p*-nitrophenol formed/min/L).

2.5. Statistical analysis

Statistical analyses were performed by SigmaStat and SigmaPlot Statistical Software ver. 11.0 (Jandel Corp., San Rafael, California).

Plasma biochemistry data were subjected to logarithmic transformation. Intergroup differences of blood parameters were assessed using *t*-test for parametric and Mann-Whitney Rank Sum Test for non-parametric data. Correlations between measured biochemical parameters were determined by Spearman Rank Order Correlation. Leukogram data, presented as mean \pm standard error of the mean (SEM), were analyzed using *t*-test and one-way ANOVA followed by the Tukey test. Student's *t*-test was used to identify differences in means, one-way ANOVA was used in order to identify differences in cell metrics, Tukey test was used to identify site and seasonal differences in leukogram readings. The *p* < 0.05 was regarded as statistically significant for all comparisons.

All data were also subjected to multivariate analysis in order to extract variables or important related information, to identify possible clusters, and to identify trends between samples and/or variables. Factor analysis (FA) was the first step in the classification process, aiding in identification of significant variables and reduction of the original data set. The next step was the Principal component analysis (PCA), usually performed by pattern recognition methods (Bosque-Sendra et al., 2012), in order to effectively reduce redundant information (Hashem Sweidan et al., 2015). Finally, the Discriminant analysis (DA) was used to evaluate the classification and to distinguish measured variables relative to seasons and sampling sites.

3. Results

3.1. Water quality and general fish health status

Physicochemical and heavy metal properties of water are presented in Tables 1 and 2. In spring in the effluent-receiving canal (sugar cane processing), Pb, Ni, Zn, Cu, suspended solids and BOD_n were the highest measured. In summer in the effluent-receiving canal (inactivity of the sugar plant), Fe, COD and COD-Mn, as well as total nitrogen and phosphorus were the highest measured. In fall in the same canal, ammonium, nitrite and nitrate were the highest measured.

Rainfall and leachate waters contributed to the total processed waters with 26.10%, 48.67% and 36.37% in spring, summer and fall, respectively.

According to the external gross signs and necropsy findings from fish captured over the seasons in the three representative water bodies, no major symptoms of diseases were evident. Gills from carp fished out of the effluent-receiving waters in all seasons showed haemorrhagic changes, associated with extensive mucus secretions and swelling. In all sampling stations and over all seasons, monogenean gill flukes were the most prevalent of all parasites. In spring and summer, the degree of monogenean infestations was greatest in fish from the County canal, while in fall it was predominant in the effluent fish. In spring, fish from the unaffected stream had a low parasitic infestation of gills with *Dactylogyrus* spp. while those from the County canal carried an intermediate load, mainly *Dactylogyrus* spp. on gills and *Thelohanellus* spp. on opercula, skin and fins. In summer, monogenean *Dactylogyrus* spp. was the only external parasite found, and only on fish in the County canal as an intermediate load. In fall, only fish from the effluent-receiving canal carried a low burden of parasites, mainly *Dactylogyrus* spp. and *Gyrodactylus* spp. on gills and skin, respectively.

3.2. Histopathology

Histopathological findings of effluent fish mostly revealed structural and functional alterations of gill tissues, related extensively to the secondary lamellae (Fig. 1), while kidney lesions related to alter-

Table 1

Physico-chemical properties of waters from water sampling sites over seasons: 1: unaffected stream, not related to any industrial nor agricultural waters; 2: canal receiving the effluent, entering the wider County canal; 3: County canal further downstream, entering the Drava river.

Parameters measured (with limits of detection)	1. Unaffected stream			2. Canal receiving the effluent			3. County canal further downstream		
	Spring	Summer	Fall	Spring	Summer	Fall	Spring	Summer	Fall
Temperature °C	13.90	17.70	13.80	18.60	16.50	13.80	16.50	22.00	9.30
Dissolved oxygen (0.1 mg O ₂ /L)	9.66	8.14	8.91	2.26	5.11	1.23	9.29	4.37	2.48
Oxygen saturation %	94.90	86.40	86.70	24.60	52.50	22.60	95.90	50.90	21.60
pH	8.13	7.94	8.08	7.89	7.70	8.05	7.65	7.31	7.50
Suspended solids (0.01 mg/L)	37.70	27.90	17.70	171.00	56.30	8.50	8.60	9.00	1.80
COD (5 mg O ₂ /L)	43.16	42.69	–	57.55	64.16	–	18.49	23.67	18.77
COD-Mn (0.01 mg O ₂ /L)	15.63	15.70	9.18	30.64	31.43	18.14	11.93	10.09	11.64
BODn (2 mg O ₂ /L)	19.85	19.00	10.75	48.26	41.66	17.50	12.84	4.73	7.60
Ammonium (0.01 mg NH ₄ -N/L)	0.02	0.36	0.49	8.40	4.30	20.00	0.05	0.59	0.52
Nitrite (0.001 mg NO ₂ -N/L)	0.01	0.03	0.027	0.40	0.85	1.48	0.04	0.01	0.077
Nitrate (0.1 mg NO ₃ -N/L)	0.30	2.60	1.30	1.30	9.30	13.40	1.20	1.80	2.90
Total nitrogen (1 mg N/L)	1.74	4.20	–	11.80	15.20	–	10.70	<5.00	<5.00
Phosphate (0.001 mg P/L)	0.09	0.20	0.10	0.97	2.30	0.63	0.09	0.20	0.83
Total phosphorus (0.001 mg P/L)	0.17	0.25	0.15	1.49	2.80	0.89	0.15	0.22	<1.00

Table 2

Heavy metals from waters over three seasons. Sampling sites: 1: unaffected stream; 2: canal receiving the effluent; 3: County canal further downstream. Limits of detection for every heavy metal measured are in parentheses in the first column.

Concentration in µg/L (with limits of detection in µg/L)	1. Unaffected stream			2. Canal receiving the effluent			3. County canal further downstream		
	Spring	Summer	Fall	Spring	Summer	Fall	Spring	Summer	Fall
Iron (1 µg/L)	8388.00	984.00	750.00	703.00	720.00	140.00	128.00	490.00	464.00
Cadmium (0.1 µg/L)	0.06	0.33	0.16	0.80	<0.20	<0.10	0.05	<0.20	0.18
Chromium (0.5 µg/L)	6.08	7.53	4.03	7.70	<1.00	<1.00	12.91	1.30	2.72
Mercury (0.2 µg/L)	<0.20	2.99	0.94	0.87	1.74	<0.20	0.45	<0.20	<0.2
Lead (1 µg/L)	1.86	4.56	5.86	5.58	0.48	1.97	2.14	0.53	2.38
Nickel (1.5 µg/L)	10.00	6.00	4.65	31.00	4.00	2.94	8.00	2.00	<1.00
Zinc (3 µg/L)	23.00	43.00	2.58	90.00	28.00	7.52	27.00	30.00	16.15
Copper (1.5 µg/L)	56.00	34.00	7.75	270.00	27.00	5.13	61.00	20.00	4.14

ations of nephrons and tubular system (Fig. 2). In effluent fish, karyolysis, binuclear hepatocytes, and distension of liver sinusoids (55% and 65% of samples, respectively) were noted (Fig. 3). Spleen alterations were noted in effluent and downstream County canal fish (Fig. 4). Fish from the downstream County canal had hyperplastic and hypertrophied gills (69%).

In spring, lamellar fusion was observed (70%), as well as spleen alterations, in form of intracellular vacuoles and granulomatous lesions in fish from the effluent (23% and 15%, respectively) and County canal (18% and 13%, respectively) (Fig. 4). In summer, the most pronounced lesions were atrophy and necrosis of lamellae (75%). Different stages of atrophy and necrosis of glomeruli were noted over seasons, with most pronounced changes in summer (55% and 45%, respectively) and fall (60% and 54%, respectively) (Fig. 2). Carp liver tissues exhibited alterations predominantly in summer and fall, mainly in effluent fish. In fall, hypertrophy of epithelial and interlamellar cells was also evident (35%).

3.3. Haematology, blood biochemistry and oxidative stress

Leukocyte counts were significantly different between the three localities and sampling seasons (Table 3). The unaffected stream fish had significantly different granulocyte numbers between spring and fall sampling seasons. At the effluent site, fish had similar leukograms across the sampling seasons. The downstream County canal fish had significant fluctuation in leukograms between sampling seasons.

In spring, total leukocyte numbers were significantly higher in fish caught in the County canal, primarily due to increased lympho-

cyte numbers when compared to fish sampled in unaffected stream and effluent. In summer, County canal fish continued to display significantly higher total leukocyte numbers, but in this season it was due to increased granulocyte numbers. During fall, fish from the unaffected stream showed significantly higher leukocyte numbers due to increased granulocyte counts.

The haematocrit values are presented in Table 4, but mostly showed no significant relationships between seasons/locations. The Spearman Rank Order Correlation demonstrated that haematocrit variables for County canal fish in summer tend to decrease while spring variables increase and *vice versa* (negative correlation coefficient and *p* value below 0.05) (see Table 5).

The values of GGT, GSH-Px and PON 1 were not measurable in most of the tested samples, probably due to haemolysis, and are therefore not presented. A general comparison of the plasma URE, CRE, TP, ALB, GLU, ALT, ALP, TRIG, CHOL, and SOD values of Prussian carp in all seasons are presented in Fig. 5. One Way Analysis of Variance established the significance (*p* < 0.05) for CHOL in the effluent-receiving canal vs. County canal in fall, and CHOL in unaffected canal in summer vs. County canal in fall. Significant differences were also noted for CRE and TRIG in the unaffected canal fish (spring vs. fall specimens), for CRE in the unaffected (spring) vs. effluent canal (fall). TRIG also had significant differences in the effluent canal (spring vs. fall specimens). The Spearman Rank Order Correlation demonstrated that pairs of variables with *p* values below 0.05 and positive correlation coefficients, such as SOD County canal (spring/summer) tend to increase together.

The input matrix for multivariate analysis consisted of 17 variables (URE, CRE, TP, ALB, GLU, ALT, ALP, TRIG, CHOL, SOD,

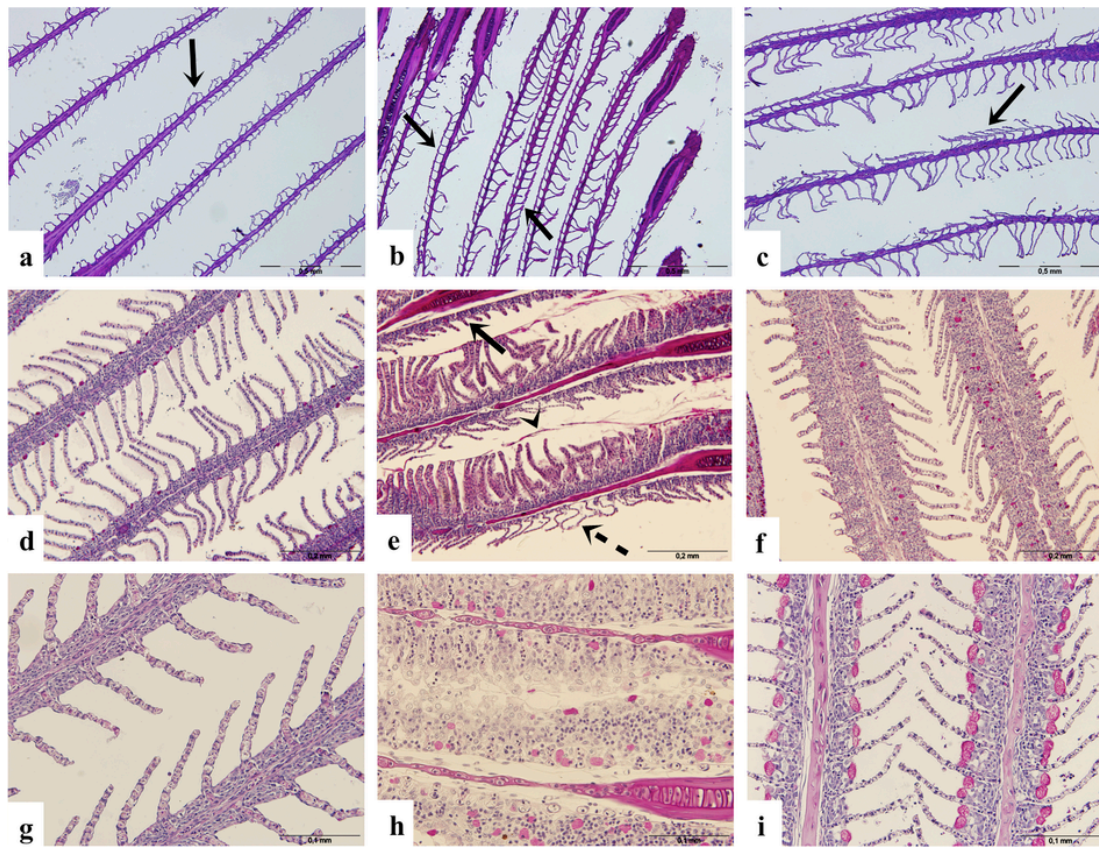


Fig. 1. Gill tissues from Prussian carp sampled in spring (a, b, c), summer (d, e, f), and fall (g, h, i). (a) Fusion of tips of neighboring lamellae pinnacles (arrow) (unaffected stream). (b) Lamellar fusion; lateral edges of fused lamellae often possessed epithelial extensions appearing to be bridging the inter-lamellar spaces (arrows) (effluent-receiving canal). (c) Lamellar curling and interlamellar bridging (arrow) (County canal). (d) Irregularities in lamellar drawing, with mucous cells uniformly distributed between medium thick interlamellar spaces (unaffected stream). (e) Severe dissociation of secondary lamellae, mainly manifested as blood congestion, damaged pilar cells and hypersecreting mucous cells (arrowhead) (effluent-receiving canal). Some specimens demonstrated shortening of secondary lamellae (arrow), as well as severe distortion (dashed arrow) (atrophy and necrosis), also on the supportive cartilaginous tissue. (f) Hypertrophy and hyperplasia of cells in interlamellar spaces and shortening/dissemination of secondary lamellae. Hyperplasia of mucous cells was noted on every examined fish (County canal). (g) Normal anatomy of gills (unaffected stream). (h) Hypertrophy of epithelial and interlamellar cells, necrosis and severe lymphocytic infiltration; intralamellar oedema and detachment from primary lamellae (effluent-receiving canal). (i) Hyperplastic and hypertrophied mucous cells in gills (County canal).

HCT, lymphocytes – LYM, polymorphonuclears – PMN, monocytes – MON, leukocytes – LEUK, length, weight), classified for each fish from different locations over seasons. Variables were reduced with the factor analysis (FA) which identified following parameters as significant for the respective location: (A) unaffected stream – URE, GLU, ALT, TRIG, SOD, HCT, LYM, PMN, LEUK; (B) canal receiving the effluent – URE, CRE, TP, ALB, GLU, ALT, ALP, TRIG, CHOL, SOD, weight; (C) County canal further downstream – TP, ALB, TRIG, CHOL, length and weight. The PCA score plots for these parameters (Fig. 6) demonstrate that the groupings were based on high percentage of the explained variance in all cases (77.07% for A, 85.63% for B, and 82.34% for C). A cross-section of observed sets of significant parameters was conducted and reduced to the following nine parameters: URE, TP, ALB, GLU, ALT, TRIG, CHOL, SOD and weight. Using those parameters, we were able to group the samples according to the fish sampling sites with the PCA showing 100% of the explained variance in all cases. Those parameters allowed their discrimination (DA) with the success of 85%, but when we used the mean values per season, the classification for locations was 100% (Fig. 7).

4. Discussion

Chemicals in the environment usually appear as complex mixtures with antagonistic, additive or synergic biological effects (Garmendia et al., 2015). In an environment where they occur in chronic and sub-lethal concentrations, changes in function and structure of fish tissues are encountered more than their mass mortality (Flores-Lopes and Thomaz, 2011). A sequence of biological responses can be triggered by multiple contaminant stress, and each of those may be used as a biomarker of effect, associated with health impairment or disease (van der Ost et al., 2003). Effluent from the WWTP under this study, discharging into a relatively small receiving canal, had a marked effect on Prussian carp community collected from the effluent canal and a downstream location (County canal). The results of the study support the hypothesis, indicating that fish biological responses related to the treated wastewater reach far downstream from the treatment plant and depend on season and/or activity of the sugar processing plant.

Volumes of wastewater produced in sugar beet processing can be considerable, with a substantial polluting potential, especially regarding the large quantity of soil and trash associated with mechanized harvesting and harvesting in wet weather, that is brought into the fac-

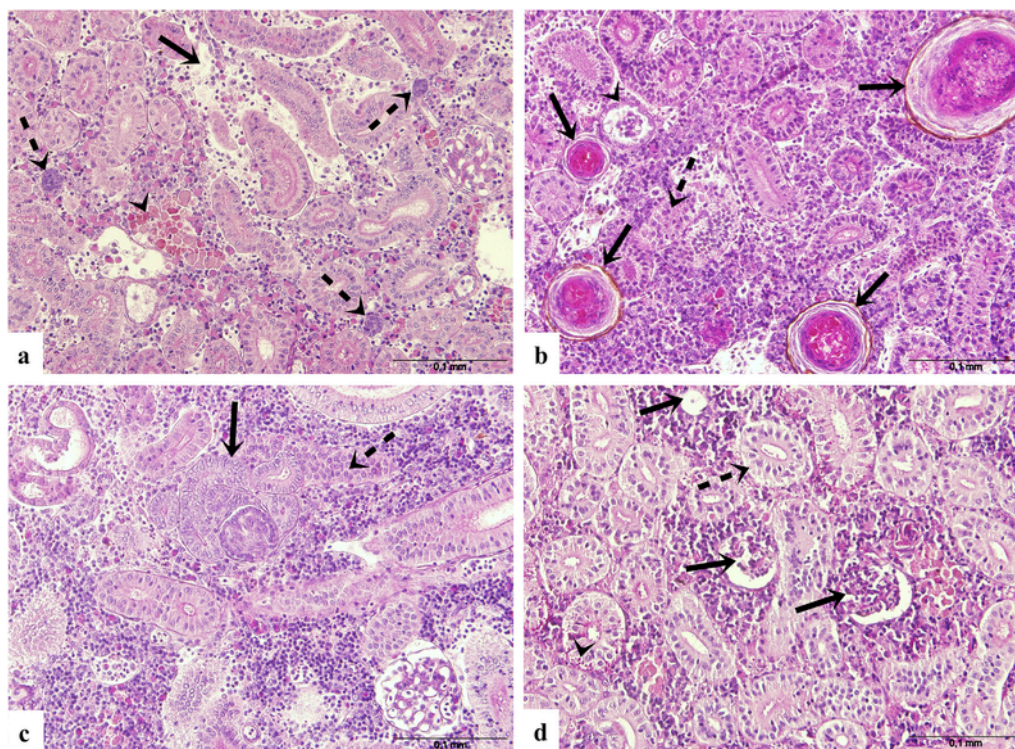


Fig. 2. Anterior kidney tissues from Prussian carp sampled in summer (a, b) and fall (c, d). (a) Extensive peritubular dilatation (arrow) and increased number of small, basophilic clusters of cells near collecting ducts (dashed arrows); increased infiltration of macrophages in-between tubules (arrowhead) (effluent-receiving canal). (b) Multifocal granulomas (arrows); darker core composed of dead cell material, melanin and bacteria, encapsulated and surrounded by macrophages; tubular degeneration and signs of diminished lumen tubules (dashed arrow); shrinkage of glomeruli and expansion of space inside Bowman's capsule (arrowhead) (County canal). (c) Developing nephrons, appearing darker (basophilic) and denser, some with reduced lumen (arrow); developing glomeruli; hyperplasia and hypertrophy of the kidney tubule cells and cloudy swelling (dashed arrow) (effluent-receiving canal). (d) Epithelial necrosis, cloudy swelling in renal tubules and presence of large eosinophilic granules inside cells (arrowhead); some areas with pycnotic nuclei and vacuoles in cytoplasm (dashed arrow); different stages of atrophy and necrosis of glomeruli (arrows) (County canal).

tory with the root crop, as a great quantity of mud enters the system through the initial beet washing and fluming process (Cheesman, 2004). Consequently, the significantly higher suspended solids, COD-Mn, BODn, and ammonium concentrations (Table 1) were measured in the effluent. Notably, the sugar cane processing did not have the same impact on the effluent waters as the sugar beet processing, mainly due to the fact that it was prewashed elsewhere prior to production. However, it particularly affected both the effluent-receiving canal and downstream County canal with heavy metal pollutants, namely Cu, Zn, Cr, Ni, all known depressants of fish immune capabilities (Javed and Usmani, 2015a). Sugar beet processing yielded the extreme ammonium, nitrate, nitrogen, phosphate concentrations in the effluent-receiving canal when related to other seasons and water bodies, and also when related to the work of El-Shafai et al. (2004). Sugar cane processing, on the other hand, resulted in high suspended solids, BOD, total nitrogen, and total phosphorus in the effluent-receiving canal. Elevated nitrogen and phosphorus may result in overgrowth of algae, decreasing the dissolved oxygen content of the water, thereby harming or killing fish and other aquatic species (Sahu and Chaudhari, 2015).

4.1. Water quality and parasitic infestations, haematology and blood biochemistry

Fish captured at the sampling sites did not vary significantly in external gross signs and necropsy findings. Downstream Prussian carp, however, suffered from a heavier parasitic infestation than fish captured in the effluent-carrying canal, which could be attributed to their possible migration over the water bodies in pursuit of the more

favorable feeding locations. Monogenean gill flukes were the most prevalent of all parasites found in this study, however they showed no site preference, also noted by Billard and Khan (2003) who established that gill parasites of fish exposed to municipal and industrial effluents had no site difference. Furthermore, Kelly and Janz (2008) found that the degree of infestation, as measured by the abundance and biomass of intestinal parasites and the abundance of monogeneans on pike (*Esox lucius*) gills, was greatest in reference fishes and intermediate in low-exposure pike, whereas high-exposure fishes harbored no parasites, which corresponds well with our findings. That could be explained by the finding that low concentrations of the WWTP pollutants are not directly toxic to the monogeneans (dactylogyrids in particular), while high concentrations result in mortality of parasites and produce a reduction of infestation levels (Siddal et al., 1997). Higher parasite infestation in fish in the downstream County canal might also be correlated with the higher density of fish in that canal when compared with the effluent fish, particularly in spring. One of the explanations is their moving to more favorable conditions, as suspended solids (in particular), COD, COD-Mn, BODn, ammonium, nitrite and total phosphorus were several-fold higher in the effluent-receiving waters, with significantly lower dissolved oxygen content. Water temperature was not significantly related with the monogenean abundance although low to intermediate infestations were noted in spring and summer. Temperature was previously shown as only one of the factors influencing proliferation of monogenean biomass, as pH, dissolved oxygen, ammonia and other water properties might mask the effect of temperature (Rakauskas and Blaevičius, 2010). There was, however, a negative correlation between monogenean prevalence and the concentration of lead in waters, particularly

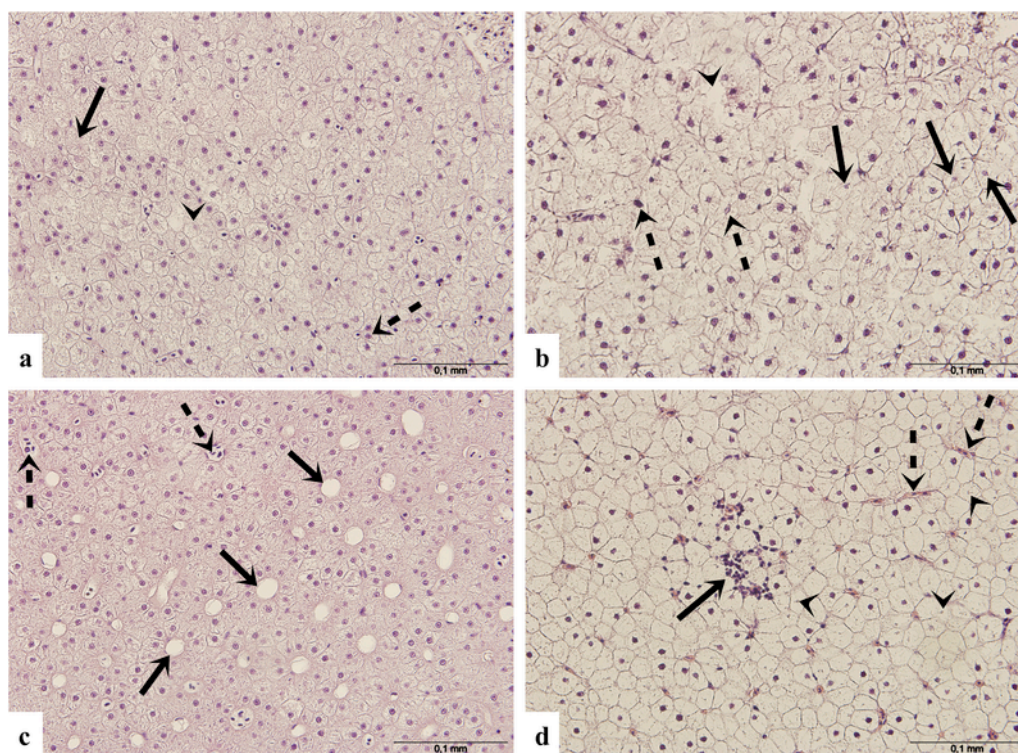


Fig. 3. Liver tissues from Prussian carp sampled in summer (a, b) and fall (c, d). (a) Densely packed hepatocytes with cloudy swelling (arrow); disappearance of hepatic cell wall, karyolysis (arrowhead) and binuclear hepatocytes (dashed arrow) (effluent-receiving canal). (b) Different stages of hepatocyte apoptosis (arrow); nuclear changes in nuclei diameter and density of chromatin in the central region (dashed arrows); focal liver necrosis (arrowhead) (County canal). (c) Severe distension or widening of sinusoids (arrows) and blood congestion (dashed arrows) (effluent-receiving canal). (d) Inflammatory leukocyte infiltration (arrow); blood cells among hepatocytes (dashed arrows); anisokaryosis and pyknotic nuclei (arrowheads) (County canal).

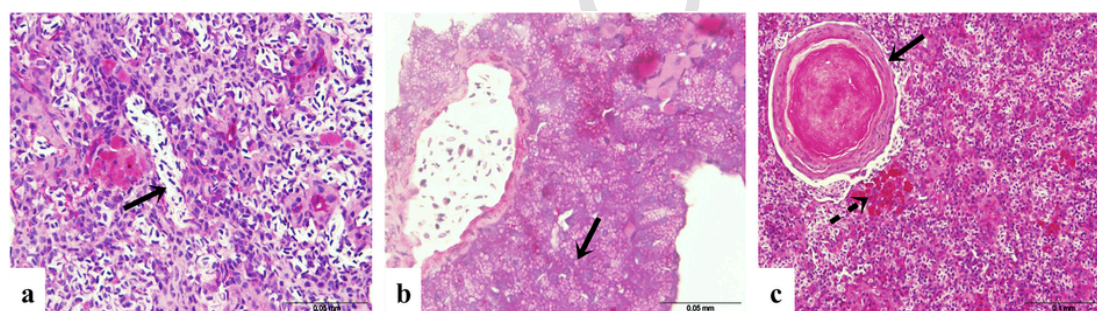


Fig. 4. Pathohistological sections of Prussian carp spleen in spring (a, b, c). (a) Normal histological structure of melanomacrophage centers (MMC) and distended sinusoids (arrow); MMC randomly distributed and well organized (unaffected stream). (b) Numerous intracellular vacuoles (effluent-receiving canal) (arrow). (c) Granulomatous lesions (arrow) and presence of increased number and size of MMC (dashed arrow) (County canal).

remarkable in spring (effluent-receiving waters, $5.58 \mu\text{g/L}$) and summer (unaffected stream, $4.56 \mu\text{g/L}$), as previously established by Bayoumy et al. (2008). The same authors also noted a significant positive correlation between monogenean infestation and concentration of nickel in the water, but we did not observe any such connection. Pollutants such as heavy metals reduce the immunological capabilities of fish host, rendering them more susceptible to parasites (Overstreet, 1993), monogenean carp infestations thus serving as auxiliary bioindicator models.

Haematology of fish, specifically leukograms, can be a valuable clinical and research tool, providing that standardized technique is applied, comparisons between non-affected control and treatment groups exist, or appropriate set of reference values is available for the species (Clauss et al., 2008). There is little available data about Prussian

carp leukocyte intervals, however reported values are in accordance with numbers observed during our study (Aksentijević, 2010; Farahi et al., 2011). Increased leukocyte (lymphocyte) counts observed in fish collected in County canal during spring sampling (highest overall Cr concentration $12.9 \mu\text{g/L}$) are also consistent with increased parasitic loads and gill pathology (Roberts, 2001). Summer samples showing overall decrease of lymphocytes, alongside relative increase of granulocytes in the effluent canal, are also indicative of neutrophilia due to heavy metal (Pb and Cd) exposure (Zelikoff, 1993; Witeska, 2005). Increased numbers of granulocytes in the County canal in summer could suggest transient neutrophilia due to stressful conditions and increased parasite loads (Alvarez-Pellitero, 2008). Generally, a fish stress leukogram reflects leukopenia with lymphopenia and granulocytosis. Such changes may persist for sev-

Table 3

Leukogram values from Prussian carp blood across seasons and localities (1: unaffected stream, not related to any industrial nor agricultural waters; 2: canal receiving the effluent, entering the wider County canal; 3: County canal further downstream, entering the Drava river). Different capital letters (A, B, C) denote significant differences among one or more evaluated parameters in locations compared between sampling seasons; different lowercase letters (a, b, c) denote significant differences among parameters between locations sampled on the same season. The same letters, or no letters, indicate there is no difference. Leukogram is presented both as a total number of counted cells per μL , and as ratio of different cell types (lymphocytes, granulocytes and monocytes) in % of total leukocyte number. The minus sign indicates no samples present or no counted cells of the respective type (unaffected stream fish in summer, monocytes and granulocytes in summer and fall, respectively).

	Spring			Summer		Fall		
	1. Stream ^A	2. Effluent ^A	3. Canal ^B	1. Effluent ^C	2. Canal ^D	1. Stream ^E	2. Effluent ^A	3. Canal ^A
Total Leukocytes (μL^{-1})	5840 \pm 809 ^a	4400 \pm 353 ^a	13400 \pm 760 ^b	5900 \pm 898 ^a	19778 \pm 1949 ^b	42840 \pm 5013 ^a	2467 \pm 565 ^b	4378 \pm 506 ^b
Lymphocytes (μL^{-1})	3840 \pm 475 ^a	3240 \pm 260 ^a	11986 \pm 740 ^b	1700 \pm 449	3022 \pm 384	5440 \pm 1029 ^a	1333 \pm 274 ^b	4378 \pm 506 ^a
%	72.5 \pm 3.0	74.2 \pm 2.7	74.1 \pm 1.6	28.8 \pm 3.7	23.5 \pm 2.0	11.6 \pm 1.2	73.2 \pm 10.9	88.7 \pm 1.8
Granulocytes (μL^{-1})	1720 \pm 265	760 \pm 92	1585 \pm 103	3900 \pm 618 ^a	16600 \pm 1826 ^b	36800 \pm 4338 ^a	1133 \pm 488 ^b	–
%	25.4 \pm 3.7	19.2 \pm 2.1	13.3 \pm 0.9	63.1 \pm 5.2	73.0 \pm 2.4	86.2 \pm 1.1	26.8 \pm 16.3	–
Monocytes (μL^{-1})	280 \pm 93	400 \pm 91	757 \pm 40	300 \pm 75	156 \pm 29	600 \pm 88	–	422 \pm 70
%	2.5 \pm 1.8	6.9 \pm 1.4	6.0 \pm 0.3	8.1 \pm 1.8	3.5 \pm 0.9	2.2 \pm 0.4	–	11.3 \pm 1.8

Table 4

Haematocrit values from Prussian carp blood across the seasons: 1: unaffected stream, not related to any industrial nor agricultural waters; 2: canal receiving the effluent, entering the wider County canal; 3: County canal further downstream, entering the Drava river.

	1. Unaffected stream	2. Canal receiving the effluent	3. County canal further downstream
Spring	33.60 \pm 14.52	42.00 \pm 8.37	47.14 \pm 7.26
Summer	29.00 \pm 13.21	27.20 \pm 18.36	37.56 \pm 10.08
Fall	36.06 \pm 11.48	27.86 \pm 13.85*	42.44 \pm 6.38*

Data are mean \pm S.D. Data with * indicate significant difference at $p < 0.05$.

eral days after the stressor is removed (Dove and Arnold, 2008). Significant decrease in granulocytes observed in fall sampling (during sugar beet processing) of fish at the effluent-receiving canal, compared to reference, could indicate immunosuppression due to poor water quality (Dunier, 1996; Arkoosh et al., 1998). Although the PCA analysis proved leukocytes, polymorphonuclears and lymphocytes as significant variables only in fish from the unaffected spring in fall, the overall observed significant differences and seasonal/location changes in the leukograms of the sampled fish were in accordance with other monitored parameters during this study.

The baseline haematocrit values of carp have been established at 28–32%, and tending to increase under stress and elevated temperatures (Hrubec and Smith, 2000; Stoskopf, 1993). The cause for increase of Hct values during acute hypoxic exposure is water loss from the blood and release of cells from depots in liver and spleen in order to increase the oxygen-carrying blood capacity (Heath, 1995). Although no major significances were found between the Prussian carp groups under survey, the highest overall Hct values were recorded in spring in downstream County canal, which coincided with the highest overall measured Cr (12.9 $\mu\text{g/L}$). Chromium was previously shown to increase Hct, serum glucose and proteins in carp (Al-Alkel and Shamsi, 1996). Fish might also increase their metabolic rate and consequently Hct values if changes in water quality or destruction of breeding niches occur (Jawad et al., 2004). Under high nitrite exposure, for example, the high activity of the methaemoglobin-reductase system converting methaemoglobin to haemoglobin results in shortening the normal life span of red blood cells, followed by an increase in haematocrit (Kroupova et al., 2005). Our effluent fish had the highest Hct values in spring (sugar cane processing), but these waters also had the highest of all measured copper concentrations (270 $\mu\text{g/L}$), similar to the findings of Mustafa et al. (2012), where the carp Hct values increased significantly in the hypoxic fish with the high Cu exposure level. The high suspended solids,

COD-Mn, BODn, and ammonium values in the effluent-receiving canal in spring (when the sugar plant was actively processing sugar cane), along with the low dissolved oxygen values, surely contributed to the impaired osmoregulation and damage to gill cells. Water quality thus caused additional stress with possible adrenergic stimulation of the erythropoietic organs causing them to contract and release stored erythrocytes into circulating blood (Mustafa et al., 2012).

Although having a high tolerance to heavy metals by a mechanism of avoidance of hypoxic status and resistance of severe plasma ions loss (Schjolden et al., 2007), elevated heavy metal concentrations (Cr 7.7 $\mu\text{g/L}$, Pb 5.58 $\mu\text{g/L}$, Ni 31 $\mu\text{g/L}$, Zn 90 $\mu\text{g/L}$, Cu 270 $\mu\text{g/L}$) might have contributed to increase of some carp plasma metabolites in our study (TRIG, CHOL, ALB, URE, TP in the effluent fish in spring). The evaluation of plasma biochemistry can provide essential data on the physiological status of fish, and fish under stress may have significant changes in plasma biochemistry (Chen et al., 2003). Blood GLU elevations may indicate a generalized stress response to a variety of environmental conditions, while a rate of change in environmental oxygen has effects on changes in plasma GLU, as hypoxic stress induces hyperglycemia (Heath, 1995). Plasma proteins coupled with GLU, TRIG and CHOL are excellent indicators of stress in fish (Wells and Pankhurst, 1999). The complex mixture of proteins in fish plasma largely originates from the liver, and some of these bind copper and zinc, although there might be a binding protein for each essential trace metal (Heath, 1995). Increased concentrations of TP can be caused by structural liver alterations (Čož-Rakovac et al., 2005). Also, as most urea in fish is produced by the liver, elevated URE is not necessarily indicative of renal disease, but is more likely associated with gill or liver impairment (Stoskopf, 1993), as noted in fish captured from the effluent in spring (sugar cane processing).

Plasma CHOL significantly varied in fall (effluent-receiving canal vs. downstream County canal), when its concentrations were the lowest and the highest of all seasons and locations, respectively. Seasonal CHOL variations in carp were not previously established (Svetina et al., 2002). Levels of CHOL in fish blood are moderately sensitive to the presence of pollutants, and seem to depend on their concentration, which affects osmoregulation. Sublethal concentrations of organic pesticides decrease CHOL in carp (Gluth and Hanke, 1985), as well as a long-term exposure of carp to mercury (Heath, 1995). Similar trend was also noted in plasma TRIG, which was the lowest in effluent fish in fall. Such a drastic drop might also be a consequence of the feed shortage in the effluent-receiving canal, confirmed by GLU and CHOL minimum values. These parameters represent the main energy metabolites often used in estimation of fish health and condition and assessment of stressors in nature (Čož-Rakovac et al., 2005). Also, such energy reserves are required by fish to mediate the effects

Table 5

Principal findings of the study over seasons/activity of the sugar plant, and three locations.

	Unaffected stream	Canal receiving the effluent	County canal further downstream
Spring (sugar cane processing)	<ul style="list-style-type: none"> - Highest overall Fe - Lowest overall monocytes - Highest overall plasma creatinine and glucose - Gill alterations: fusion of lamellae pinnacles (moderate) 	<ul style="list-style-type: none"> - Highest overall suspended solids, BODn - Highest overall Pb, Cd, Ni, Zn, Cu - Highest overall plasma albumins, total proteins and triglycerides - Gill alterations: fusion of lamellae, lamellar bridging (severe) - Spleen alterations: melanomacrophage centers (severe) 	<ul style="list-style-type: none"> - Highest overall Cr - Highest overall lymphocytes, monocytes, haematocrit - Highest overall plasma SOD - Highest overall monogenean infestation - Gill alterations: lamellar curling and inter-lamellar bridging (severe) - Spleen alterations: granulomas, melanomacrophage centers (severe)
Summer (sugar plant inactive)	<ul style="list-style-type: none"> - Gill alterations: irregularities in lamellar structure, abundance of mucous cells (mild) 	<ul style="list-style-type: none"> - Highest overall COD, COD-Mn, total nitrogen, phosphate, total phosphorus - Highest overall plasma urea, ALT; lowest plasma SOD - Gill alterations: dissociations of secondary lamellae, blood congestions, damaged pillar cells, abundance of mucous cells (severe) - Kidney alterations: peritubular dilatations, basophilic clusters, macrophage infiltration of tubules (severe) - Liver alterations: disappearance of cell walls, karyolysis and binuclear hepatocytes (severe) 	<ul style="list-style-type: none"> - Gill alterations: shortened secondary lamellae, atrophy, necrosis (moderate) - Kidney alterations: multifocal granulomas, tubular degenerations, diminished lumen (moderate) - Liver alterations: hepatocyte apoptosis, nuclear changes (moderate)

Table 5 (Continued)

	Unaffected stream	Canal receiving the effluent	County canal further downstream
Fall (sugar beet processing)	<ul style="list-style-type: none"> - Highest overall water temperature - Highest overall total leukocytes and granulocytes 	<ul style="list-style-type: none"> - Highest overall ammonium, nitrite, nitrate concentration; lowest overall dissolved O₂ - Lowest overall total leukocytes, lymphocytes and granulocytes - Highest overall plasma ALP; lowest plasma TRIG - Gill alterations: hypertrophy of epithelial and interlamellar cells, necrosis, lymphocytic infiltration (severe) - Kidney alterations: developing nephrons, reduced lumen, hyperplasia and hypertrophy of tubules, cloudy swelling of tubule cells (severe) - Liver alterations: distention of sinusoids and blood congestion (severe) 	<ul style="list-style-type: none"> - Lowest overall O₂ saturation - Highest overall plasma cholesterol - Gill alterations: hyperplastic and hypertrophied mucous cells (severe) - Kidney alterations: epithelial necrosis, cloudy swelling of tubule cells, atrophy and necrosis of glomeruli (moderate) - Liver alterations: inflammatory leukocyte infiltration, anisokaryosis and pyknotic nuclei (moderate)

of stress and serve as energy buffers (Topić Popović et al., 2012). Therefore, given the decline in these values, the immune suppression and the weakening of the innate, non adaptive immune defense system of carp in the effluent-receiving waters in fall is very likely (Javed and Usmani, 2015). However, since ALT and ALP activities were not elevated in the effluent-receiving canal, the deleterious effects might have been of transient nature, as semi-permeable plasma membranes prevented the leakage of enzymes from damaged (liver) tissues (Topić Popović et al., 2006).

In spring, a significant decrease in SOD was noted in the fish from the effluent-receiving canal. Since SOD is one of the main antioxidative enzymes serving to detoxify reactive oxygen species (ROS), a decrease in its activity changes the redox status of the cells, and can be attributed to the inhibition of the superoxide radical formation (Ural, 2013) implying the reduced ability to protect cells against superoxide radicals. The municipal wastewaters were shown to decrease SOD activity, reflecting the damage to the SOD protein due to ROS overproduction (Karadag et al., 2014). In this work SOD activity had a seasonal pattern since the SOD variables in the County canal tended to increase together in spring and in summer, while in summer SOD tended to decrease as the fall SOD increased and *vice versa*. The highest overall SOD values measured were in spring and summer in County canal fish downstream. In chronic experiments antioxidant enzyme activities are usually increased in aquatic organisms, and high SOD activity indicates that fish could protect itself against the toxic effect of superoxide anion radical (Huang et al., 2007). Indeed, upon exposure of zebrafish (*Danio rerio*) to phenol pollutants, the trend of SOD activity was at first elevated, while with the adaptation to the concentration over time it gradually restored

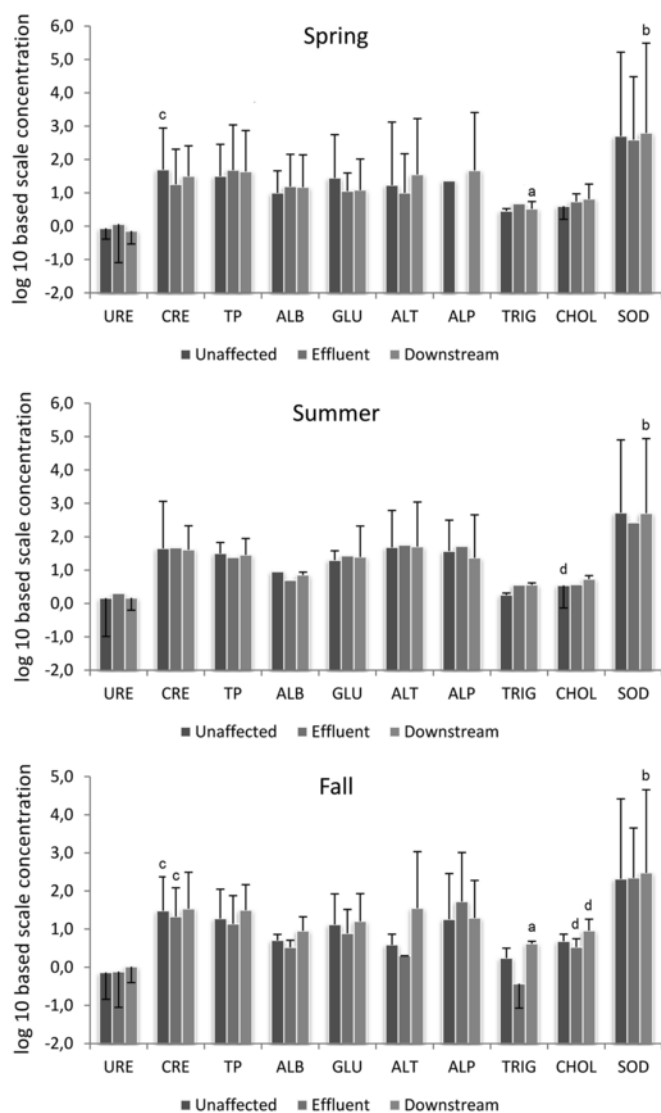


Fig. 5. Plasma biochemistry and oxidative stress parameters in Prussian carp over seasons. URE (urea), CRE (creatinine), TP (total proteins), ALB (albumin), GLU (glucose), ALT (alanine aminotransferase), ALP (alkaline phosphatase), TRIG (triglyceride), CHOL (cholesterol), and SOD (activity of superoxide dismutase) are presented. Concentration data are log-transformed (y-axis). The error bars represent the standard deviation. The differences between the parameters marked with identical lowercase lettering (a,b,c,d) are statistically significant ($p < 0.05$).

(Zhang et al., 2014). Also, it is not unlikely that organic contaminants may be measured at the most downstream sites from the WWTP (Jeffries et al., 2010), which indicates cumulative inputs of such compounds in the rivers, and surely impacts fish organismic indicators of tissue damage.

4.2. Water quality and histopathology

The complex matrix of the WWTP effluent under study varied seasonally and spatially, impacting the fish tissue responses, particularly evident from the gill structure. The severity of gill tissue alterations varied, and increased from spring till fall. Elevated ammonium levels in the effluent-receiving canal (spring 8.4 mg/L, fall 20 mg/L) might have reduced excretion and result in an uptake of ammonia into the tissues, not only gills, but also kidney and liver (Tetreault et al., 2012). Significant reduction in fish growth, irregular gill ventila-

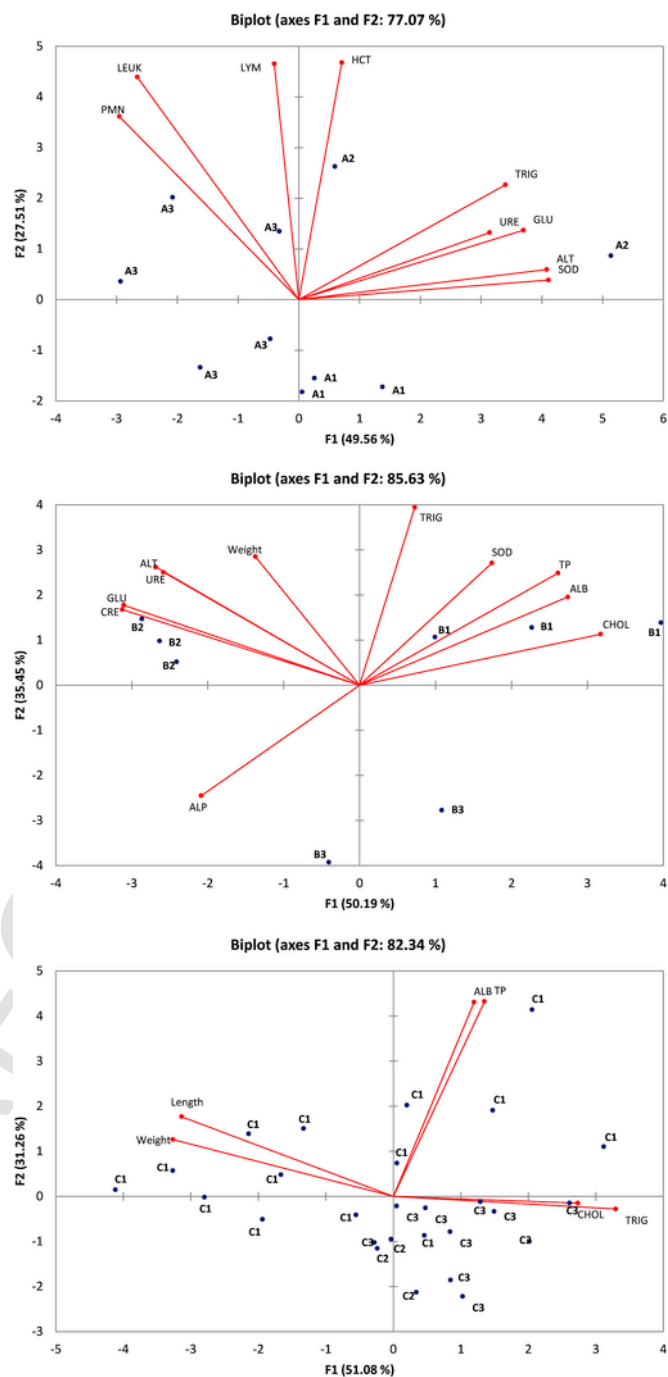


Fig. 6. Principal component analysis (PCA) for plasma biochemistry and oxidative stress parameters in Prussian carp from the unaffected stream (A), canal receiving the effluent (B) and County canal further downstream (C), over seasons (Spring: 1; Summer: 2; Fall: 3). Fish caught in spring in the unaffected stream (A1) grouped in the fourth quadrant, mostly on the mean values of URE, GLU, ALT, TRIG, SOD, and HCT which significantly differed from the mean values in other two seasons. Summer carp (A2) displayed ALT and SOD as dominant variables, while fall values (A3) grouped on the left side of the plot with significantly different means of PMN, LEUK and LYM. Effluent fish (B) grouped according to the season based on 11 parameters, 10 of which were responsible for grouping of spring and summer parameters, ALP significantly varying in fall (B3). PCA identified only 6 parameters as significant in County canal fish (C), with fish length and weight as significant in spring (C1), and TRIG and CHOL in fall (C3).

tion and morphology, hyperplasia of gill epithelium and lamellae have been shown in fish chronically exposed to environmentally rele-

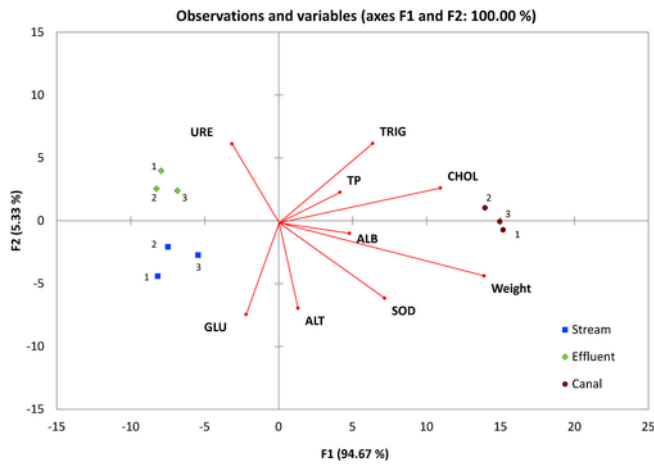


Fig. 7. Classification of Prussian carp based on mean values of biometry, haematology, plasma biochemistry and oxidative stress parameters according to the sampling sites and over seasons (spring: 1, summer: 2, fall: 3), by use of Discriminant analysis (DA). Classification is showing 100% of the explained variance of significant cases, comprising of the first and second components. The first component in classification was dominant (94.67%) with dominance variables of CHOL, SOD and weight of fish.

vant ammonium concentrations (Tetreault et al., 2012). In fish under this assay, gills of effluent fish in general exhibited slight to severe changes that included epithelial hyperplasia with lamellar fusion, epithelial hypertrophy, and proliferation of mucous cells. Such gill alterations were noted by others (Marioara et al., 2009; Parvathi and Sarsu, 2011; Nikalje et al., 2012) reporting them as typical gill lesions in response to a prolonged exposure to a wide range of heavy metals. Gills are a major target for metal toxicity and also the main point of entry for metals. Metals enter the apical and basolateral membranes of the specialized ion-transporting chloride cells in mostly divalent forms, such as Co^{2+} , Zn^{2+} , Cd^{2+} (Olsson et al., 1998). As chloride cells are the primary site of calcium uptake, there is a competition between Ca and divalent metal ions for metal uptake. Thus increasing water hardness and acidified freshwaters reduce heavy metal toxicity (Ghosh and Adhikari, 2006). Exposure to toxicants, particularly heavy metals, leads to rapid inhibition of ion-translocating enzymes, such as Na^+/K^+ , and Ca^{2+} -ATPase, as well as exchangers and ion channels (Di Giulio and Hinton, 2008). When the metal-biotic ligand complex, which encompasses sodium or calcium channel proteins, reaches a critical concentration, fish mortality occurs (Di Toro et al., 2001). Although the physiological mechanism of gill toxicity varies with various metals, biotic ligand models of acute metal toxicity may predict specific toxicity for individual species (Smith et al., 2015).

In the effluent fish in summer and fall (sugar beet processing), damages of gill structure included shortenings of secondary lamellae, atrophy and inflammatory processes with necrosis. Similar findings have been reported from environmental monitoring studies (Barišić et al., 2015). However, bridging of the neighboring secondary lamellae was one of the most pronounced changes in fish gills. Under acute exposure to heavy metals, bridging tends to reduce the total surface contact area between contaminated water flow and bloodstream (Topić Popović et al., 2015a). It is known that both soluble and suspended heavy metals are readily absorbed across fish gills causing deleterious changes in tissue structures, particularly Cu (Mustafa et al., 2012). In this study, Cu was extremely elevated (270 $\mu\text{g/L}$) in effluent-receiving canal in spring (sugar cane processing), and together with elevated suspended solids (171 mg/L) and other heavy metals (Cr 7.7 $\mu\text{g/L}$, Pb 5.58 $\mu\text{g/L}$, Ni 31 $\mu\text{g/L}$, Zn 90 $\mu\text{g/L}$), as well as hy-

poxic conditions (2.26 $\text{mg O}_2/\text{L}$) contributed to changes in gill morphology. The restricted lumen of the gill vessels forces red blood cells to move into reduced space, thus giving rise to further cellular deformations (Mustafa et al., 2012).

Liver is a target organ for lesions by pollutants due to its high metabolic activity in xenobiotic metabolism, excretion, digestion and storage (Bernet et al., 2000). The severity of the liver tissue damage in this study varied in relation to seasons and locations, as in the work of Kessabi et al. (2014), where it also varied in response to contaminants with bioaccumulation character which altered the liver function and led to gradual degeneration of cells. The detected dilations of sinusoids, found in livers of our effluent fish in fall, are presumed to be a consequence of long-term blood congestions and blockage of sinusoids, which widened to facilitate the flow of metabolites and detoxicants (Nikalje et al., 2012). Vacuolar structures in livers of those Prussian carp, filled with cellular debris, might be a response of detoxifying Kupffer cells to pollutants (Omar et al., 2013). Liver and gill lesions of fish from the effluent-receiving canal in spring could be linked with high plasma URE concentrations ($1.15 \pm 0.07 \text{ mmol/L}$) of these fish, as well as high ammonium concentrations (8.40 mg/L), as in the work of Bernet et al. (2000) where polluted waters were associated with high blood urea concentration and liver and gill damage. The observed inflammatory clusters between hepatic lobules might indicate a reaction to bacterial stimuli, such as *Aeromonas* spp. isolated from water and liver tissues in our previous work (Topić Popović et al., 2015b). Liver changes in effluent and downstream fish in fall (sugar beet processing) in the form of distension of sinusoids, blood congestion, leukocyte infiltration, blood cells by hepatocytes, anisokaryosis and picnotic nuclei suggest high hepatocytes metabolic activity in response to the uptake of heavy metals (Omar et al., 2013). Also, vacuolar degeneration of spleen, found in the effluent fish, was assigned as an indicator of pollution with heavy metals (Georgieva et al., 2010). However, due to relatively normal ALT and ALP activities in fish from the effluent-receiving canal through all seasons, ($20.5 \pm 14.85 \text{ mmol/L}$, $23.21 \pm 24.45 \text{ mmol/L}$ respectively, liver function was apparently preserved.

The signs of severe damage of the renal tissue and renal function due to heavy metal toxicity were reported from various fish species (Omar et al., 2013). Most prominent changes in this work, observed in effluent fish in summer (the highest overall Fe concentration, 720 $\mu\text{g/L}$), were the increased number of basophilic clusters; while in fall (Fe 464 $\mu\text{g/L}$) newly developed nephrons prevailed, along with epithelial necrosis and atrophy, eosinophilic granules and vacuoles in cytoplasm. According to Cormier et al. (1995) developing nephrons and basophilic clusters might serve as general indicators of ecological condition through nephrotoxicity at specific sites. Signs of necrosis and damage to kidney tubules, on the other hand, might be due to accumulation of inflammatory cells associated with metal toxicity (Tetreault et al., 2012). In spring, (sugar cane processing), when most of the measured heavy metals were significantly increased, elevated CRE levels of $26 \pm 11.31 \text{ mmol/L}$ indicate impairment of kidney function, particularly glomerular insufficiency and disruption of carbohydrate metabolism (Omar et al., 2013). Kidney and gill tissue alterations might impair the ability of Prussian carp to survive and reproduce normally (Tetreault et al., 2012).

5. Conclusions

Although the avoidance response is one form of the phenotypic adaptation allowing fish to survive in the altered environment (Vosyliene et al., 2003), Prussian carp under investigation demonstrated a high capacity to live in waters of diminished quality with

ability to compensate for environmental changes caused by treated wastewaters of municipal, hospital and industrial origin. The area of WWTP influence on Prussian carp biological responses, measured by the toxic effects on blood and tissue parameters, reaches far downstream and is not of a localized nature.

Prussian carp displayed responses and changes in physiological parameters with the most prominent effects on gill and liver cell damage and blood SOD activity, while CHOL and TRIG showed the strongest correlation with the season and sugar plant (beet processing) activity. Alterations of tissue morphology appear to be a consequence of loss of cellular and structural tissue integrity, as the degree of structural heterogeneity of fish organs was enhanced with increasing concentrations of heavy metals in the environment. These changes can be correlated with the oxidative stress levels and neutrofilia. Integrated histopathological, haematological and biochemical findings, however non specific to one particular agent, could thus serve as valuable biomarkers for native fish adaptive patterns and monitoring of water quality and pollution of freshwater ecosystems. The application of multivariate tools allowed discrimination of the most relevant predictors which outline unified fish biological responses to the effluent relative to location and seasons. It was also found valuable in other studies in monitoring the impact of complex mixtures of toxicants in aquatic ecosystems on fish species, and controlling conditions needed in validating and monitoring of fish health (Hashem Sweidan et al., 2015; Dzul-Caamal et al., 2016).

In aquatic environments impacted with complex contaminants acting synergistically, causal relationships between biological responses and environmental stressors should be interpreted. Thus, a set of risk analysis and risk management measures is recommendable for the area under study, particularly in the form of hazard identification, effect assessment, exposure assessment, characterization of risk and regulatory measures. They are of particular importance since deleterious effects of multiple stressors on (fish) populations are challenging to detect in native populations, which tend to be under constant or recurring exposure causing a prolonged physiological response. To that end, the research of early-warning signals (biomarkers), reflecting the adverse biochemical or cellular responses towards anthropogenic pollution, measurable in body fluids, cells or tissues (van der Ost et al., 2003), is aimed to indicate the effects at higher biological levels. Integrated histopathological, haematological and biochemical analyses, as biomarkers of effect, yielded information on biological effects of mixture of pollutants in “real world exposure”, and helped in assessment of the magnitude of impact of treated wastewater on native fish. We therefore recommend the use of coupled biomarkers as a tool for improving ecological risk assessment and biomonitoring of environmental pollution.

Conflict of interest

The authors declare no conflict of interest.

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