Environmental Management
DOI 10.1007/s00267-017-0964-7

Effects of Multi-Component Mixtures from Sewage Treatment Plant Effluent on Common Carp (*Cyprinus carpio*) under Fully Realistic Condition

Pham Thai Giang[1] · **Viktoriia Burkina**[1] · **Sidika Sakalli**[1] · **Heike Schmidt-Posthaus**[2] · **Martin Krøyer Rasmussen**[3] · **Tomas Randak**[1] · **Roman Grabic**[1] · **Katerina Grabicova**[1] · **Ganna Fedorova**[1] · **Olga Koba**[1] · **Oksana Golovko**[1] · **Jan Turek**[1] · **Daniel Cerveny**[1] · **Jitka Kolarova**[1] · **Vladimir Zlabek**[1]

Received: 28 June 2017 / Accepted: 9 November 2017

Abstract This study characterized changes in biomarker responses in common carp (*Cyprinus carpio*) upon exposure to effluent water discharged from a sewage treatment plant (STP) under real conditions. Fish were exposed to contamination in Cezarka pond, which receives all of its water input from the STP in the town of Vodnany, Czech Republic. Five sampling events were performed at day 0, 30, 90, 180, and 360 starting in April 2015. In total, 62 pharmaceutical and personal care products (PPCPs) were detected in the polar organic chemical integrative sampler. Compared to a control pond, the total concentration of PPCPs was 45, 16, 7, and 7 times higher in Cezarka pond at day 30, 90, 180, and 360, respectively. The result of oxidative stress and antioxidant enzyme biomarkers indicated alterations in the liver and intestine tissues of fish from Cezarka pond at day 30 and 360, respectively. High plasma vitellogenin levels were observed in both exposed females (180 and 360 days) and males (360 days) compared with their respective controls. However, only exposed female fish had higher vitellogenin mRNA expression than the control fish in these periods. Exposed female fish showed irregular structure of the ovary with scattered oocytes, which further developed to a vitellogenic stage at day 360. Low white blood cell levels were indicated in all exposed fish. Despite numerous alterations in exposed fish, favorable ecological conditions including high availability of food resulted in a better overall condition of the exposed fish after 1 year of exposure compared to the controls.

Keywords Biological pond · Biological effects · Endocrine disruption · Integrate biomarker response

Abbreviations

ALT	Alanine aminotransferase
ALB	Albumins
ALP	Alkaline phosphatase
NH^3	Ammonia
AST	Aspartate aminotransferase
Ca^{2+}	Calcium
CAT	Catalase
CK	Creatine kinase
RBC	Red blood cell
FFPW	Faculty of Fisheries and Protection of Water
GLU	Glucose
GR	Glutathione reductase
GST	Glutathione S-tranferase
GPx	Glutathione peroxidase
PCV	Hematocrit value
Hb	Hemoglobin concentration
PHOS	Inorganic phosphate
LACT	Lactate
LDH	Lactate dehydrogenase

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00267-017-0964-7) contains supplementary material, which is available to authorized users.

The original version of this article was revised: The authors' given and family name was corrected.

✉ Pham Thai Giang
phamthai@frov.jcu.cz

[1] Faculty of Fisheries and Protection of Waters, South Bohemian Research Centre of Aquaculture and Biodiversity of Hydrocenoses, University of South Bohemia in Ceske Budejovice, Zatisi 728/II, 389 25 Vodňany, Czech Republic

[2] Vetsuisse Faculty, Centre for Fish and Wildlife Health, Department of Infectious Diseases and Pathobiology, University of Bern, Laenggassstrasse 122, 3001 Bern, Switzerland

[3] Department of Food Science, Aarhus University, P.O. Box 50, DK-8830 Tjele, Denmark

Published online: 20 November 2017

WBC	White blood cell
Mg	Magnesium
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean erythrocyte volume
NSAIDs	Non-steroidal anti-inflammatory drugs
POCIS	Polar organic chemical integrative sampler
STPs	Sewage treatment plants
PPCPs	Pharmaceutical and personal care products
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TP	Total proteins
TRIG	Triglycerides
VTG	Vitellogenin

Introduction

Sewage treatment plant (STP) effluents have been shown to contain a wide spectrum of chemical contaminants, such as pharmaceutical and personal care products (PPCPs), pesticides, and other contaminants originating from households, industry, and agriculture (Calisto and Esteves 2009; Halling-Sørensen et al. 1998; Köck-Schulmeyer et al. 2012). The discharge of effluents from STPs has several detrimental effects on the health of aquatic ecosystems, including the aquatic environment and aquatic organisms. These effects include nutrient imbalance (Björn Gücker 2006), behavioral changes (Garcia-Reyero et al. 2011; Schoenfuss et al. 2002), and disruption of the endocrine pathways of aquatic organisms (Anway et al. 2005; Mills and Chichester 2005).

The presence of PPCPs and their impact on the aquatic environment have received increasing concern (Zenobio et al. 2015). Environmentally relevant concentrations of PPCPs are much lower than the corresponding therapeutic doses used for medical treatments, and acute toxicity tests have often failed to detect the subtle action elicited by drugs at low dosage (Fent et al. 2006). Furthermore, PPCPs are regularly detected in the complex mixtures of unrelated molecules with diverse chemical structures, persistence, specificity, and biological activity (Fent et al. 2006; Lopez-Serna et al. 2012). The joint ecotoxicity of such chemical cocktails is typically higher than the toxicity of each individual compound (Cleuvers 2003; Eguchi et al. 2004; Kortenkamp 2009). In particular, even if the levels of compounds in the mixture are only present below their respective toxicity thresholds, a joint toxic effect cannot be ruled out. Therefore, it is challenging to predict the effects of such mixtures on fish health. Neglecting the potential impacts of chemical cocktails could possibly result in underestimating the actual effect of PPCPs under real conditions, which depend on the concentration, number of compounds, and their modes of action.

There is increasing evidence of the presence, distribution, and effects of PPCPs (Ebele et al. 2017). However, a significant proportion of the data are derived from laboratory experiments. To the best of our knowledge, data from laboratories are properly used as reference data. However, these data may fail to reflect the effects in environmental conditions due to underestimation of the additive, synergistic, or antagonistic interactions during chronic exposure to mixtures of hundreds of compounds. Their interactions with other stressors such as temperature or pH are also unclear.

Several approaches have been applied in toxicological studies to understand the effect of pollutants mixtures in environmental conditions, such as field and cage studies. However, each method has its own disadvantages. In fact, information about the bioaccumulation and biomagnification of toxic compounds may be impossible to gather in cage studies because the organisms do not consume natural food. Furthermore, randomly caught fish in field studies may yield invalid results because of the migration of fish. Without information about the origin and history of fish, it is almost impossible to determine the effects and their time variations.

In this study, an experiment was performed in a biological pond that receives input from only STP effluent under fully realistic conditions. This scenario represents a common condition in central Europe, where treated communal wastewater is released to a recipient pond via biological and production ponds. The fish were stocked in an STP recipient pond in order avoid the stress of caging and to ensure natural feeding conditions as well as environmental interactions.

The common carp (*Cyprinus carpio*) is one of the most economically important freshwater species in Europe (Bostock et al. 2016). This specie has been widely used as a model to monitor the effects of pollutant compounds and water quality in both laboratory and field conditions (Dobsikova et al. 2006; Gungordu 2011; Witeska and Wakulska 2007; Zivna et al. 2016). The gills, liver (Gonzalez-Gonzalez et al. 2014; Thibaut et al. 2006), and blood (Islas-Flores et al. 2013) have high xenobiotic metabolizing. Biochemical changes in these tissues have been used as effective indicators of pollutant exposure in fish.

To describe sequential responses, the focus was on the early biomarker signals (oxidative stress), later responding parameters (blood parameters, endocrine disruption) and chronic effects (histology). The response of enzyme activities and ionoregulatory has previously been shown to change in the gills, liver, and blood of common carp exposed to PPCPs (Gonzalez-Gonzalez et al. 2014; Saravanan et al. 2011). Despite low environmental

Fig. 1 Map of the Vodnany sewage treatment plant, control, and Cezarka ponds

concentrations, PPCPs have a wide range of modes of action. Therefore, a large set of biochemical responses that can reflect both subtle and evident changes is needed to investigate the effect of PPCP mixtures on fish (He et al. 2011; van der Oost et al. 2003).

Oxidative stress and antioxidant enzymes are among the sensitive parameters that change in the presence of PPCPs. The induction of lipid peroxidation (LPO) and glutathione S-tranferase (GST) has been detected in the gills (Li et al. 2011b) and digestive tissues (Brandao et al. 2013) of fish exposed to carbamazepine. Changes in superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities were detected in the brain, gills, and liver of common carp exposed to a mixture of diclofenac and acetaminophen (Nava-Álvarez et al. 2014).

Hematological and biochemical plasma parameters are important indices for assessing the physiological status of fish and toxicological symptoms (Rao 2006a; Velisek et al. 2011). Vitellogenin (VTG) is an important and effective endocrine disruption biomarker (Hansen et al. 1998) that has been proven to have increase in fish exposed to certain pollutants (Paraso et al. 2017; Petrovic et al. 2002). Histology has commonly been used to define the toxicological effects and is considered as a gold standard (Kilty et al. 2007).

This study investigated the effects of a complex mixture of chemicals predominated by PPCPs from STP effluent. Common carp was exclusively exposed to the effluent of an STP for a period of 360 days in a biological pond to assess the effects in realistic conditions.

Material and Methods

Standard and Reagents

Liquid chromatography–mass spectrometry (LC/MS)-grade acetonitrile and methanol (Lichrosolv, Hypergrade) were obtained from Merck (Darmstadt, Germany). Formic acid (LC/MS grade) was obtained from Fisher Scientific (USA). All other chemicals were obtained from Sigma-Aldrich (Europe).

Experimental Area

The study areas were Cezarka pond and a control pond (Fig. 1). Cezarka pond (2.6 ha) is a biological pond designed for the retention of treated effluent from the Vodnany STP in the Czech Republic. Vodnany (population 7000) is a town that is adjacent to the Blanice River in South Bohemia. The commercial activity in Vodnany consists of light industry (poultry slaughter, manufacturing of agricultural machinery) along with intensive agriculture and horticulture in the surrounding area. Cezarka belongs to a cascade of aquaculture ponds that are connected to the

Blanice River. Moreover, the pond is suitable for the breeding of common carp. Sewage water treatment in the STP facility involves primary mechanical filtration and sedimentation followed by activated sludge treatment.

The control pond (0.12 ha) was selected from the pond system of the Faculty of Fisheries and Protection of Water (FFPW), University of South Bohemia, Vodnany, Czech Republic. The pond was chosen as an ecological representative for the water bodies in the region. The control pond is about 2 km away from Cezarka pond and is in the same range of geography and weather. Although it was different in size from the Cezarka pond, the depth and fish density were kept similar. Similar to other water bodies in the region, the control pond receives water from the Blanice River upstream from the town.

Sampling Sites and Field Deployment of the Polar Organic Chemical Integrated Sampler (POCIS)

Water pollutants in the control and Cezarka ponds were monitored using both grab and passive samplers. POCISs were deployed for a period of 10 days prior to each fish sampling event. Passive and grab water samplers were collected at three locations in Cezarka (near the inlet, near the outlet, and in a middle location) and in one location in the control pond (in a middle location). The analyses of polar compounds from pesticide configuration of POCIS-Pest were performed according to the procedures of Grabic et al. (2010, 2012). Briefly, exposed samplers were cleaned and disassembled, and the sorbent was transferred to glass chromatographic columns.

The analytes targets were eluted with 50 ml of methanol, dichloromethane, and toluene (1:8:1 v/v/v). The extraction solvent was then changed to methanol, and samples were analyzed using LC-MS/MS. The target analytes were separated and detected using a TSQ Quantum triple-stage quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) coupled with an Accela 1250 LC pump (Thermo Fisher Scientific, San Jose, CA, USA) and HTS XT-CTC autosamplers (CTC Analytics AG, Zwingen, Switzerland). An analytical Hypersil GOLD aQ column (50 mm length, 2.1 mm i.d, 5-µm particles; Thermo Fisher Scientific) was used to chromatographically separate the target analytes. The dates of sampling events and the sampling points are shown in Supplementary Material 1.

Fish

Both male and female common carp were obtained from a local facility (66 ± 3 g body weight and 170 ± 0.3 mm in length). The carp were randomly stocked in the control and experimental ponds with similar relative stock density (0.14 fish/m^2) in April 2015. Both ponds were harvested, and all of the remaining fish were removed prior to stocking. The fish ate only natural food in both ponds. The trophic level of the control pond is typical for an average aquaculture pond in the region.

Sampling took place after 0, 30, 90, 180, and 360 days of exposure. Additional information about the sampling times is shown in Supplementary Material 1. The fish were collected from both ponds by electrofishing. The fish were handled according to the national and institutional guidelines for the protection of human subjects and animal welfare and the Law Against Animal Cruelty (082/2002-V2). Approval was obtained from Czech National Directive No. 419/2012 for the protection of experimental animals.

Twelve individual fish from each pond were sampled at each time point. Blood samples were taken from each fish by puncturing the caudal vein using a syringe with heparin as an anticoagulant at a concentration of 5000 IU/ml of heparin sodium salt. The fish were then sacrificed by severing the spinal cord. Their weight and length were then measured. Organs including the gills, gonads, liver, intestine, and white muscle were quickly removed. Blood plasma was obtained by centrifuging the blood samples in a cooled centrifuge (4 °C, 10,000 × g, 10 min) and stored at −80 °C until analysis. A small volume of blood was immediately used to determine hematological variables (Svobodova et al. 2012). The liver, muscle, intestine, and gills were dissected and stored at −80 °C for biochemical analysis. At each sampling point, samples of the kidneys, livers, gills, and gonads of fish from both ponds were also fixed in 10% formalin for histological examination.

Morphological Indices

The body, liver, and gonad weights were recorded for each fish, in addition to each animal's length. The condition factor (CF), hepatosomatic index (HSI), and gonadal somatic index (GSI) were calculated for each fish according to the literature (White and Fletcher 1985):

$$\mathrm{CF} = \frac{b}{L^3} \times 100 \qquad \mathrm{HSI} = \frac{1}{b} \times 100 \qquad \mathrm{GSI} = \frac{g}{b}\mathrm{x}100,$$

where b is the body weight (g), L is the total length (cm), l is the liver weight (g), and g is the gonad weight (g).

Histological Examination

Fixed samples of the gills, kidney, liver, spleen, and gonads of exposed and control fish were embedded in paraffin and cut with a microtome into 4-µm sections for histology. The sections were stained with haematoxylin-eosin (H&E) and examined by light microscopy. The organs were excised and placed longitudinally in a capsule to ensure the largest possible cut section for each organ. Due to the relatively

small size of samples, one cut section per fish and organ was examined, and the lesions were assumed to be distributed equally throughout the tissue. Pathological changes were graded as 0 (none), 1 (minimal), 2 (mild), 3 (mild to moderate), 4 (moderate), 5 (moderate to severe), or 6 (severe) relative to the normal structures described in healthy animals. After a first screening, the following criteria were selected for semiquantitative evaluation: gills: epithelial hyperplasia, lamellar fusion, parasitic infestation; liver: hepatocyte vacuolation, pericholangiar inflammation, perivascular inflammation, granuloma, vessel wall degeneration; gonads: sex, differentiation stage, uniformity of germ cells, oocyte degeneration.

Biochemical Assays of Fish Tissues

The post-mitochondrial supernatant (PMS) was obtained as described by Howcroft et al. (2009). GST activity was determined using 1-chloro-2,4-dinitrobenzene as a substrate according to the method of Habig et al. (1974), which was adapted for a microplate reader by Frasco and Guilhermino (2002). CAT activity was determined using the method of Claiborne (1985) by measuring the decrease in hydrogen peroxide in a 96-well flat-bottom ultraviolet-transparent microtiter plate.

SOD activity was determined using the method of Nishikimi et al. (1972). This assay relies on the ability of the enzyme to inhibit the phenazine methosulfate-mediated reduction of nitro blue tetrazolium (NBT) dye. Glutathione reductase (GR) activity was determined using the method of Cribb et al. (1989) with some modifications using 50 µl of PMS (approx. 0.2 mg/ml) and 150 µl of reaction solution. GPx activity was measured using the method of Mohandas et al. (1984). Oxidative damage was assessed by determining the level of LPO, which was measured as thiobarbituric acid reactive substances (TBARS). This was carried out using the methodology of Ohkawa et al. (1979). The details of each method are presented in Supplementary Material 2.1.

Biochemical Assays in Fish Blood Plasma

A VETTEST 8008 analyzer (IDEXX Laboratories Inc., USA) was used according to the manufacturer's instructions to determine biochemical indices, including glucose (GLU), total proteins (TP), ammonia (NH_3), aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin (ALB), creatine (CREA), lactate dehydrogenase (LDH), creatine kinase (CK), lactate (LACT), phosphorous (PHOS), magnesium (Mg), triglyceride (Trig), alkaline phosphatase (ALP), and calcium (Ca^{2+}).

Vitellogenin

Gene expression of VTG in liver tissue

RNA isolation was carried out using the Trizol method (Rio et al. 2010). The isolated RNA was converted to cDNA by reverse transcription using an iScript cDNA synthesis kit (Bio-Rad, Canada), according to the manufacturer's instructions. cDNA was diluted in RNAase free water and stored at 20 °C until further use. Semi-quantitative PCR was conducted according to the method of Rasmussen et al. (2011) using TaqMan probes. Primers and TaqMan probes were designed with Primer Express 3.0.1 using common-carp-specific sequences of genomic DNA. The primers and probes are shown in Supplementary Material 2.2.1.

The relative mRNA expression was calculated by relating the obtained values for threshold cycles to a standard curve obtained by running a serial dilution of one cDNA sample. The mRNA expression was normalized to the mRNA expression of beta actin and expressed as arbitrary units. The expression of beta actin did not significantly differ between the experimental and control groups. The average of the control groups according to exposure time was arbitrarily set to 1, and the experiment groups were expressed relative to the corresponding control group. The details of this method are presented in Supplementary Material 2.2.

Concentration of VTG in blood plasma

The concentration of VTG in blood plasma was determined using a Biosense Elisa test kit for carp (Biosense, Norway), according to the instructions of the manufacturer. Principally, the test utilizes specific binding between antibodies and VTG to quantify the VTG concentration in samples. The wells of microplates were pre-coated with a specific capture antibody that binds to VTG in standards and samples added to the wells. A different VTG-specific detecting antibody was added to create a sandwich of VTG and antibody, which was detected by an enzyme-labeled secondary antibody. The enzyme activity was determined by adding a substrate that yields a colored product, and the color intensity was directly proportional to the amount of VTG present.

Hematological Parameters

Transformation solution (0.1 g of potassium ferricyanide, 0.025 g of potassium cyanide, 0.07 g of potassium dihydrogenphosphate, and up to 0.5 l of distilled water) was used to determine the hemoglobin (Hb) concentration. The indices tested were determined by methods described by Svobodova et al. (2012) and included red blood cells

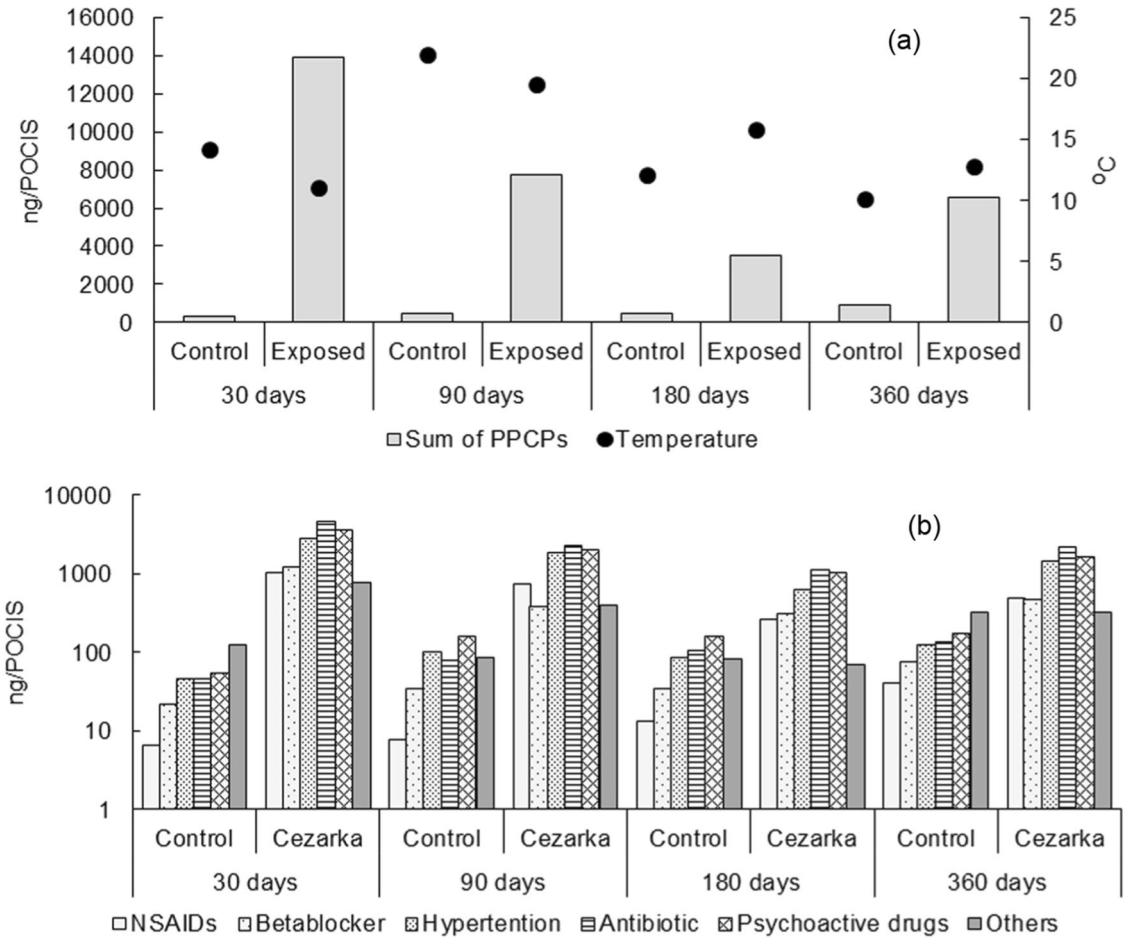

Fig. 2 Sum of pharmaceutical and personal care products (PPCPs) in polar organic compounds integrated samplers (POCISs) (column) ($n = 3$) and average temperature (dot) (**a**); and the total PPCPs according to groups—NSAIDs (Non-steroidal anti-inflammatory drugs), beta blocker, hypertension, psychoactive compound, antibiotic, others (fibrate, azole, antihistamine)—in the control and Cezarka ponds (**b**)

(RBCs), hematocrit (PCV), mean erythrocyte volume (MCV), mean erythrocyte hemoglobin (MCH), and mean color concentration (MCHC). The level of Hb was determined spectrophotometrically at 540 nm (Helios Epsilon, UNICAM). The MCV and MCHC values were obtained from blood count analysis as conventional biomarkers. The procedures were based on unified methods for hematological examinations of fish (Svobodova et al. 2012).

Integrated Biomarker Response (IBR)

For better understanding of the overall effect of STP effluent on fish, the IBR was calculated according to Beliaeff and Burgeot (2002). The final IBR values were calculated by dividing the number of biomarkers (n) based on the suggestion of Broeg and Lehtonen (2006). The necessary results of the data standardization procedure for the IBR calculation are presented in star plots. The IBR index was calculated using the results of LPO (measured as TBARs), SOD, CAT, GPx, GR, and GST.

Statistical Analysis

Differences within the sampling period were tested between the exposed group and the corresponding control group in CF, HSI, GSI, mRNA expression, VTG, oxidative stress and antioxidant enzymes, blood parameters, and histopathology. First, the data were tested for normality using the Shapiro–Wilk test. If the normality condition was satisfied, the Student's unpaired t-test was used to determine whether there were any significant differences between the control and exposed groups within sampling period. If the normality condition was not satisfied, a nonparametric Mann–Whitney U-test was used. A significant difference was recognized when $P < 0.05$ was found. All of statistical analyses were performed using SPSS statistical software (version 23, IBM Corp.) for Windows.

Results

At the day 0, 30, and 90, the fish were juveniles. Therefore, all parameters were investigated without considering sex. At 180 and 360 days, the fish were separated into males and females to see the sex differences in the respective parameters.

Occurrence of Selected Compounds in Water

Figure 2a shows the results of water temperature and total PPCPs identified using POCIS in the control and Cezarka ponds. At 30, 90, 180, and 360 days, the water temperature in Cezarka pond was 3.1 °C lower, 2.4 °C lower, 3.6 °C higher, and 2.6 °C higher than in the control pond, respectively. The total concentrations of 62 detected PPCPs ranged from 305 to 880 ng/POCIS in the control pond and from 3490 to 14000 ng/POCIS in Cezarka pond. The total PPCPs increased slightly over time in the control pond, and a sinusoidal trend was noted in the Cezarka pond. The total PPCPs concentration was highest after 30 days. After that point, it constantly decreased until 180 days before suddenly spiking up by 360 days. Non-steroidal anti-inflammatory drugs (NSAIDs) (6.6–1020 ng/POCIS), beta-blockers (22–1210 ng/POCIS), hypertension drugs (47–2770 ng/POCIS), antibiotics (48–4590 ng/POCIS), and psychoactive drugs (55–3580 ng/POCIS) were the most abundant drug compounds in both ponds (Fig. 2b). The pharmaceuticals irbesartan, telmisartan, carbamazepine, and tramadol were found in relatively high concentrations in all of the sampling events in Cezarka pond. The average concentrations of these compounds were 1990, 1440, 512, and 422 ng/POCIS, respectively. The full list of measured concentrations of analyzed compounds is presented in Supplementary Material 4.

Morphological Indices

The morphological indices of common carp from the control and Cezarka ponds are shown in Table 1. Information about the mean full length and body weight of the fish are presented in Supplementary Material 5. Within a year, the CF value was highest at 90 days with 66 and 92% increases compared with day 0 in the control and Cezarka ponds, respectively (Table 1). The relationship between fish body weight and total body length was 25, 34, and 52% higher in fish from Cezarka pond compared with fish in the control pond at 30, 90, and 360 days.

In the control pond, the mean HSI was higher at day 30 compared with day 0, and we noted a decreasing trend of HSI at other time points. Significant differences between the control and exposed fish were observed in the sampled fish at 30 (33), 90 (57), and 180 (20%) days. Although both male and female-exposed fish had lower HSI than the

Table 1 Effect of long-term exposure to STP effluent on CF, HSI, and GSI of common carp in control and Cezarka pond

Time	0 days	30 days	90 days	180 days			360 days		
Fish	Juvenile ($n = 12$)	Juvenile ($n = 12$)	Juvenile ($n = 12$)	♀ ($n = 5$)	♂ ($n = 7$)	♀ and ♂ ($n = 12$)	♀ ($n = 6$)	♂ ($n = 6$)	♀ and ♂ ($n = 12$)
Condition factor									
Control	1.31 ± 0.01	1.94 ± 0.08	2.17 ± 0.07	2.25 ± 0.26	2.10 ± 0.13	2.16 ± 0.13	1.94 ± 0.52	1.92 ± 0.36	1.93 ± 0.04
Exposed		2.19 ± 0.06*	2.51 ± 0.05*	2.10 ± 0.07	2.26 ± 0.84	2.18 ± 0.06	2.36 ± 0.13*	2.52 ± 0.58*	2.45 ± 0.06*
Hepatosomatic index									
Control	4.32 ± 0.21	5.97 ± 0.25	2.59 ± 0.10	2.75 ± 0.13	2.77 ± 0.12	2.76 ± 0.09	3.87 ± 0.09	4.29 ± 0.33	4.12 ± 0.20
Exposed		3.95 ± 0.15*	4.09 ± 0.34*	2.54 ± 0.11	1.85 ± 0.20*	2.20 ± 0.15*	3.34 ± 0.50	3.61 ± 0.32	3.50 ± 0.27
Gonad somatic index									
Control	NA	0.16 ± 0.03	0.13 ± 0.03	0.50 ± 0.13	3.13 ± 0.59	2.17 ± 0.56	0.37 ± 0.08	2.64 ± 0.49	1.72 ± 0.44
Exposed		0.25 ± 0.03	1.21 ± 0.47*	2.17 ± 0.85*	6.61 ± 1.90	4.67 ± 1.24	2.29 ± 0.47*	9.41 ± 0.77*	6.44 ± 1.21*

NA not available

Note: Data are presented as mean ± SEM; an asterisk corresponds to significant differences compared to control value (*$P < 0.05$)

Table 2 Effect of STP effluent on the level of TBARS (μmol g of wet tissue^{-1}), GST (μmol min^{-1} mg protein^{-1}), CAT (μmol min^{-1} mg protein^{-1}), SOD (μmol NBT min^{-1} mg protein^{-1}), GR (nmol min^{-1} mg protein^{-1}), and GPx (nmol min^{-1} mg protein^{-1}) in the liver, gill, intestine and muscle of common carp tissues

Time points		0 days	30 days	90 days	180 days			360 days		
Fish		Juvenile ($n = 12$)	Juvenile ($n = 12$)	Juvenile ($n = 12$)	♀ ($n = 5$)	♂ ($n = 7$)	♀ and ♂ ($n = 12$)	♀ ($n = 6$)	♂ ($n = 6$)	♀ and ♂ ($n = 12$)
Liver										
CAT	Control	2.16 ± 0.20	1.61 ± 0.20	0.54 ± 0.07	0.98 ± 0.07	0.98 ± 0.13	0.98 ± 0.08	1.55 ± 0.06	1.47 ± 0.08	1.50 ± 0.05
	Exposed		0.74 ± 0.04*	0.80 ± 0.13	1.07 ± 0.15	0.56 ± 0.10*	0.82 ± 0.12	1.32 ± 0.06	1.44 ± 0.07	1.39 ± 0.05
SOD	Control	2.29 ± 0.17	2.53 ± 0.74	6.60 ± 0.10	4.13 ± 0.30	3.89 ± 0.42	3.99 ± 0.27	3.94 ± 0.31	3.99 ± 0.39	3.97 ± 0.25
	Exposed		4.40 ± 0.67*	5.65 ± 1.23	2.87 ± 0.56*	4.68 ± 0.75	3.78 ± 0.52	3.90 ± 0.26	3.54 ± 0.35	3.69 ± 0.23
GPx	Control	0.38 ± 0.03	0.51 ± 0.06	0.17 ± 0.02	0.29 ± 0.04	0.34 ± 0.07	0.32 ± 0.04	0.26 ± 0.04	0.22 ± 0.03	0.24 ± 0.03
	Exposed		0.23 ± 0.03*	0.25 ± 0.04	0.38 ± 0.06	0.21 ± 0.04	0.30 ± 0.04	0.17 ± 0.04	0.21 ± 0.05	0.19 ± 0.03
GR	Control	0.07 ± 0.01	0.08 ± 0.01	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.00
	Exposed		0.03 ± 0.00*	0.03 ± 0.00	0.04 ± 0.01	0.06 ± 0.00	0.04 ± 0.01	0.04 ± 0.01	0.06 ± 0.00*	0.05 ± 0.00*
GST	Control	1.96 ± 0.19	1.66 ± 0.21	0.42 ± 0.07	1.29 ± 0.13	1.51 ± 0.26	1.42 ± 0.16	3.08 ± 0.23	2.62 ± 0.13	2.81 ± 0.14
	Exposed		0.65 ± 0.07*	1.44 ± 0.29*	1.84 ± 0.26	1.29 ± 0.34	1.56 ± 0.22	3.22 ± 0.43	4.20 ± 0.17*	3.79 ± 0.29*
TBARs	Control	7.67 ± 0.69	7.98 ± 2.01	30.95 ± 3.97	15.95 ± 1.21	17.64 ± 2.22	16.94 ± 1.36	6.10 ± 1.00	6.53 ± 0.43	6.35 ± 0.46
	Exposed		8.58 ± 1.84*	36.26 ± 11.8	13.41 ± 2.02	23.14 ± 5.58	18.28 ± 1.84	8.67 ± 3.88	7.04 ± 1.21	7.72 ± 1.68
Gill										
CAT	Control	0.32 ± 0.02	0.32 ± 0.04	0.29 ± 0.02	0.26 ± 0.01	0.31 ± 0.05	0.30 ± 0.03	0.15 ± 0.06	0.22 ± 0.04	0.19 ± 0.03
	Exposed		0.30 ± 0.02	0.25 ± 0.02	0.22 ± 0.02	0.18 ± 0.02	0.20 ± 0.02*	0.24 ± 0.07	0.27 ± 0.06	0.24 ± 0.04
SOD	Control	2.03 ± 0.07	1.53 ± 0.08	1.08 ± 0.09	0.95 ± 0.30	1.41 ± 0.17	1.22 ± 0.17	4.64 ± 0.23	5.13 ± 0.69	4.93 ± 0.37
	Exposed		1.68 ± 0.09	1.75 ± 0.06*	2.08 ± 0.31	1.91 ± 0.40	2.00 ± 0.24*	5.42 ± 0.69	4.43 ± 0.30	4.84 ± 0.35
GPx	Control	0.30 ± 0.01	0.27 ± 0.01	0.36 ± 0.02	0.27 ± 0.03	0.28 ± 0.01	0.28 ± 0.01	0.16 ± 0.04	0.10 ± 0.02	0.13 ± 0.02
	Exposed		0.32 ± 0.02*	0.28 ± 0.01*	0.22 ± 0.01	0.21 ± 0.01*	0.21 ± 0.01*	0.12 ± 0.02	0.09 ± 0.02	0.11 ± 0.01
GR	Control	0.53 ± 0.02	0.41 ± 0.03	0.28 ± 0.02	0.26 ± 0.03	0.25 ± 0.02	0.25 ± 0.02	0.10 ± 0.01	0.14 ± 0.02	0.12 ± 0.01
	Exposed		0.35 ± 0.02	0.24 ± 0.01	0.20 ± 0.02	0.20 ± 0.02	0.20 ± 0.01*	0.13 ± 0.02	0.14 ± 0.01	0.14 ± 0.01
GST	Control	1.97 ± 0.08	1.63 ± 0.09	1.04 ± 0.06	0.93 ± 0.05	1.06 ± 0.03	1.00 ± 0.03	2.41 ± 0.11	2.13 ± 0.03	2.25 ± 0.09
	Exposed		1.56 ± 0.07	1.48 ± 0.06*	1.29 ± 0.09	0.91 ± 0.05	1.10 ± 0.08	2.20 ± 0.09	2.11 ± 0.07	2.15 ± 0.05
TBARs	Control	6.22 ± 0.74	9.62 ± 1.15	11.17 ± 1.55	11.09 ± 1.26	14.1 ± 4.04	12.84 ± 2.37	0.42 ± 0.13	0.88 ± 0.21	0.69 ± 0.15
	Exposed		8.18 ± 1.24	8.23 ± 0.89	10.07 ± 2.79	8.51 ± 2.08	9.29 ± 1.67	1.49 ± 0.28*	3.34 ± 1.08*	2.57 ± 0.68*
Intestine										
CAT	Control	0.71 ± 0.04	0.56 ± 0.07	0.61 ± 0.07	0.30 ± 0.04	0.38 ± 0.04	0.34 ± 0.04	0.44 ± 0.04	0.41 ± 0.03	0.43 ± 0.02
	Exposed		0.84 ± 0.08*	0.53 ± 0.05	0.34 ± 0.04	0.32 ± 0.05	0.33 ± 0.03	0.30 ± 0.06	0.26 ± 0.06*	0.28 ± 0.04*
SOD	Control	3.11 ± 0.30	1.25 ± 0.15	1.84 ± 0.23	1.97 ± 0.38	2.01 ± 0.29	1.99 ± 0.22	4.18 ± 1.68	6.31 ± 0.45	5.43 ± 0.77
	Exposed		1.30 ± 0.23	1.88 ± 0.19	2.32 ± 0.38	2.21 ± 0.23	2.26 ± 0.21	5.99 ± 2.03	5.66 ± 1.11	5.81 ± 1.04
GPx	Control	0.27 ± 0.23	0.20 ± 0.04	0.16 ± 0.01	0.14 ± 0.02	0.18 ± 0.03	0.17 ± 0.02	0.96 ± 0.21	0.46 ± 0.21	0.67 ± 0.16

Table 2 continued

Time points		0 days	30 days	90 days	180 days			360 days		
Fish		Juvenile ($n = 12$)	Juvenile ($n = 12$)	Juvenile ($n = 12$)	♀ ($n = 5$)	♂ ($n = 7$)	♀ and ♂ ($n = 12$)	♀ ($n = 6$)	♂ ($n = 6$)	♀ and ♂ ($n = 12$)
	Exposed		0.17 ± 0.02	0.15 ± 0.02	0.13 ± 0.01	0.18 ± 0.02	0.15 ± 0.01	0.17 ± 0.06*	0.16 ± 0.05	0.17 ± 0.04*
GR	Control	0.47 ± 0.10	0.52 ± 0.05	0.27 ± 0.02	0.21 ± 0.02	0.27 ± 0.02	0.25 ± 0.02	0.01 ± 0.00	0.00	0.00
	Exposed		0.41 ± 0.02	0.29 ± 0.02	0.28 ± 0.01	0.28 ± 0.04	0.28 ± 0.02	0.00	0.00	0.00
GST	Control	1.93 ± 0.15	1.60 ± 0.18	1.83 ± 0.16	1.34 ± 0.07	1.54 ± 0.09	1.46 ± 0.06	0.81 ± 0.11	1.05 ± 0.14	0.95 ± 0.10
	Exposed		1.36 ± 0.15	2.03 ± 0.26	1.74 ± 0.08*	1.8 ± 0.23	1.80 ± 0.12*	0.96 ± 0.24	0.96 ± 0.09	0.96 ± 0.11
TBARs	Control	4.47 ± 1.25	4.38 ± 0.49	2.73 ± 0.39	2.18 ± 0.44	2.97 ± 0.40	2.64 ± 0.31	3.52 ± 0.50	4.50 ± 0.86	3.76 ± 0.36
	Exposed		5.37 ± 1.38	4.07 ± 0.48	4.53 ± 1.09	4.63 ± 0.74	4.58 ± 0.63	3.93 ± 0.51	5.02 ± 0.49	4.80 ± 0.44
Muscle										
CAT	Control	0.37 ± 0.05	0.35 ± 0.05	0.28 ± 0.03	0.26 ± 0.06	0.26 ± 0.04	0.26 ± 0.03	0.08 ± 0.03	0.17 ± 0.04	0.13 ± 0.03
	Exposed		0.27 ± 0.03	0.23 ± 0.05	0.24 ± 0.04	0.39 ± 0.06	0.31 ± 0.04	0.11 ± 0.05	0.09 ± 0.01	0.10 ± 0.02
SOD	Control	0.96 ± 0.08	1.00 ± 0.15	1.10 ± 0.06	1.99 ± 0.25	1.36 ± 0.15	1.62 ± 0.12	3.66 ± 0.24	3.89 ± 0.11	3.80 ± 0.63
	Exposed		1.54 ± 0.18*	1.38 ± 0.18	1.78 ± 0.20	1.25 ± 0.14	1.52 ± 0.14	3.27 ± 0.98	5.76 ± 2.10	4.72 ± 1.30
GPx	Control	0.31 ± 0.01	0.33 ± 0.01	0.32 ± 0.02	0.28 ± 0.01	0.35 ± 0.01	0.32 ± 0.01	1.04 ± 0.66	0.85 ± 0.54	0.93 ± 0.40
	Exposed		0.33 ± 0.01	0.34 ± 0.01	0.34 ± 0.02	0.37 ± 0.02	0.35 ± 0.01	1.48 ± 0.97	0.55 ± 0.39	0.94 ± 0.46
GR	Control	0.12 ± 0.01	0.16 ± 0.02	0.09 ± 0.01	0.07 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
	Exposed		0.12 ± 0.01	0.12 ± 0.03	0.08 ± 0.01	0.11 ± 0.02	0.09 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
GST	Control	0.74 ± 0.04	1.06 ± 0.15	0.98 ± 0.15	0.80 ± 0.11	1.04 ± 0.13	0.94 ± 0.09	0.23 ± 0.05	0.21 ± 0.08	0.22 ± 0.05
	Exposed		0.97 ± 0.04	1.30 ± 0.35	0.78 ± 0.11	1.10 ± 0.10	0.94 ± 0.09	0.51 ± 0.04*	0.58 ± 0.11*	0.55 ± 0.07*
TBARs	Control	2.21 ± 0.48	1.86 ± 0.56	1.58 ± 0.34	1.04 ± 0.16	1.10 ± 0.15	1.07 ± 0.11	5.12 ± 0.90	5.17 ± 0.42	5.15 ± 0.42
	Exposed		1.60 ± 0.20	0.99 ± 0.12*	1.97 ± 1.08	1.43 ± 0.17	1.70 ± 0.53*	6.06 ± 0.46	5.03 ± 0.67	5.46 ± 0.44

Note: Data are means ± SEM. An asterisk corresponds to significant differences between exposed and corresponding control group (*$P < 0.05$)

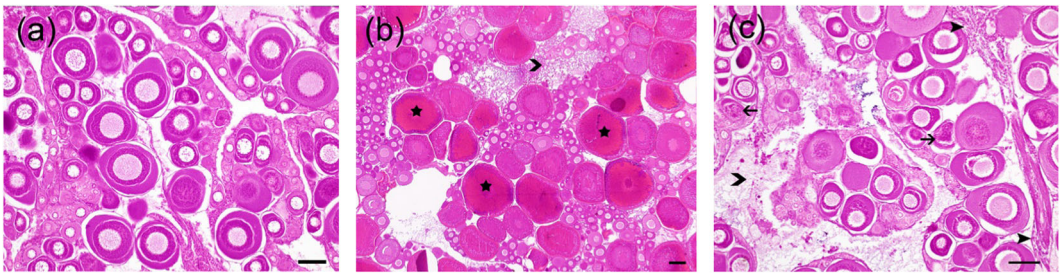

Fig. 3 Histopathology (H&E stain) of fish ovaries in the control (**a**) and the Cezarka ponds (**b, c**) after 360 days of exposure. **a** Ovary of a control female, oocytes up to previtellogenic stage; **b** ovary of an exposed female, oocytes further developed up to vitellogenic stage (stars), multifocal interstitial edema (open arrowhead); **c** ovary of an exposed female, irregular structure with interstitial edema (open arrowheads) and multifocal infiltration with lymphocytes and macrophages (closed arrowheads), multiple oocytes degenerated (arrow). The scale bar corresponds to 50 μm

control fish at 180 days, the reduction was significant in only male fish.

The GSI of fish from Cezarka pond was significantly higher than in the control group at 90 and 360 days. The GSIs of both males and females in the control pond decreased over the period of 180–360 days, and the GSI of fish exposed to STP effluent discharge constantly increased in both sexes. Although the GSIs of both male and female-exposed fish were higher than control at 180 days, the elevation was significant in only females, resulting in no significant difference when including both sexes. At 360 days, the GSI levels in both male and female-exposed fish were higher than in control fish.

Oxidative Stress and Antioxidant Responses

LPO levels (measured as TBARs) and four antioxidant enzymatic reactions (CAT, SOD, GR, and GPx) were measured in the liver, gills, intestine, and muscle tissues. Changes in these levels are summarized in Table 2 for each tissue type. In the liver, the levels of TBARs, CAT, SOD, GR, and GPx in fish exposed to STP effluent were significantly different from those of control fish after 30 days. STP effluent-exposed carp showed a significant increase in hepatic TBARs level and SOD activities (by 2.3-fold and 1.7-fold, respectively) and a decline in hepatic CAT, GR, and GPx (by 2.2-fold, 3.2-fold, and 2.2-fold, respectively). Additionally, GR activity in the exposed fish was significantly higher than in the control fish at day 360. Although both male and female-exposed fish had higher GR levels than control fish at this sampling time, the elevation was significant in only male fish.

In the gills, only the GPx level was significantly elevated in fish exposed in Cezarka pond for 30 days. At day 90, significantly lower GPx and higher SOD activities were observed in the gills of exposed fish compared with the control fish. Significantly lower levels of activity of CAT, GR, and GPx (by 1.4-fold, 1.2-fold, and 1.3-fold, respectively) and higher levels of SOD (by 1.6-fold) in the gills of exposed fish were observed when compared with the control fish at day 180. The TBAR level in the gills was significantly higher at 360 days of exposure. However, no significant differences were found in terms of antioxidant enzyme activities in this period. At 180 days, both male and female-exposed fish had higher levels of SOD and lower levels of GPx, GR, and CAT than control fish. However, the only significant differences were in the elevation of SOD in females and the reduction of GPx in males (Table 2).

In the intestine, a significantly higher level of CAT activity was found in Cezarka fish on day 30, but the level was lower after 360 days of STP exposure. The activity of GPx was significantly lower on day 360 of exposure. At 360 days, both male and female-exposed fish had lower levels of CAT and GPx, but the reductions were significant in only males for CAT and females for GPx (Table 2).

In the muscles, only SOD activity was significantly elevated in exposed fish on day 30. The TBAR levels exhibited significantly larger differences from the controls on day 90 and 180.

Glutathione-S-Transferase Activity

Table 2 shows the changes in the GST activity in different fish tissues after long-term exposure to STP effluent. Hepatic GST activity exhibited a bi-phasic variation in exposed fish, indicating a significant decrease in GST activity at 30 days and an increase at 90 and 360 days. The GST activity in the gills, intestine, and muscle tissues of effluent-exposed fish was significantly higher than in the control fish at 90 (by 1.4-fold), 180 (by 1.24-fold), and 360 (by 2.6-fold) days.

Histopathology

The results of the histopathological examination are shown in Fig. 3. The gonads of control and effluent-exposed male fish were in the same developmental stage (up to sperms) after 360 days of exposure. In contrast, the ovaries of STP effluent-exposed females were more advanced compared

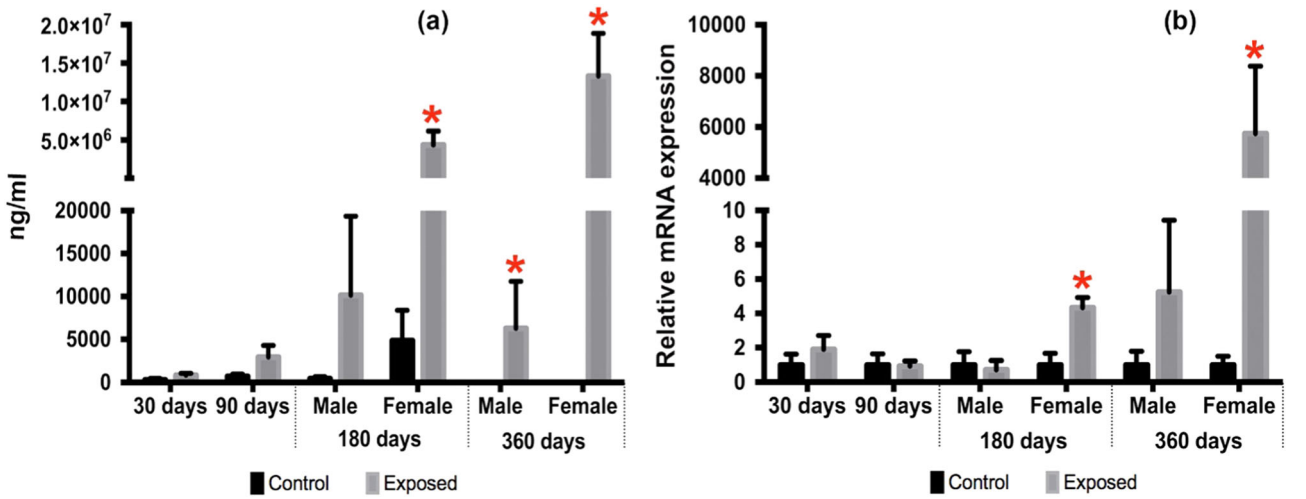

Fig. 4 Effect of STP effluent on the concentration of vitellogenin (VTG) in blood plasma ($n = 12$) (**a**) and VTG relative mRNA expression in liver (**b**) ($n = 10$). Data are means ± SEM, $n = 12$. An asterisk corresponds to significant differences between exposed and corresponding control group ($^*P < 0.05$)

Table 3 Effect of STP effluent on hematological parameters in fish at day 90, 180, and 360 from the control and Cezarka ponds

Time points	90 days	180 days			360 days		
Fish	Juvenile ($n = 12$)	♀ ($n = 5$)	♂ ($n = 7$)	♀ and ♂ ($n = 12$)	♀ ($n = 6$)	♂ ($n = 6$)	♀ and ♂ ($n = 12$)
PCV (l/l)							
Control	0.40 ± 0.01	0.31 ± 0.10	0.33 ± 0.20	0.33 ± 0.01	0.26 ± 0.1	0.32 ± 0.20	0.29 ± 0.01
Exposed	0.33 ± 0.02*	0.32 ± 0.10	0.42 ± 0.2*	0.37 ± 0.02	0.30 ± 0.20	0.36 ± 0.10	0.34 ± 0.01*
Hb (g/l)							
Control	84.1 ± 2.8	57.7 ± 1.6	60.0 ± 3.2	59.0 ± 2.0	50.5 ± 2.5	63.4 ± 3.7	58 ± 3.0
Exposed	71.1 ± 4.4*	57.4 ± 1.3	72.9 ± 4.3*	65.1 ± 3.2	55.7 ± 3.5	71.4 ± 2.9	64.8 ± 3.1
RBC (T/l)							
Control	1.31 ± 0.03	1.48 ± 0.04	1.45 ± 0.06	1.46 ± 0.04	1.23 ± 0.07	1.49 ± 0.06	1.38 ± 0.06
Exposed	1.14 ± 0.06*	1.11 ± 0.05*	1.47 ± 0.10	1.29 ± 0.08	1.27 ± 0.08	1.39 ± 0.06	1.34 ± 0.05
WBC (G/l)							
Control	185.3 ± 20.1	116.0 ± 20.4	91.4 ± 14.8	101.7 ± 12.1	100.9 ± 9.4	78.9 ± 11.0	88.1 ± 7.9
Exposed	84.3 ± 7.8*	42.6 ± 4.1*	37.3 ± 5.1*	39.9 ± 3.2*	28.9 ± 5.6*	18.7 ± 2.5*	23.0 ± 2.9*
MCV (fl)							
Control	302.7 ± 8.9	210.8 ± 6.4	231.1 ± 11.6	222.6 ± 7.6	213.0 ± 11.2	212.6 ± 6.7	212.8 ± 5.8
Exposed	296.6 ± 16.4	293.4 ± 13.5*	292.1 ± 24.8	292.8 ± 13.4*	239.1 ± 3.9	261.9 ± 8.1*	252.4 ± 5.9*
MCH (pg)							
Control	64.8 ± 3.2	39.0 ± 1.7	41.5 ± 2.0	40.4 ± 1.3	41.2 ± 1.6	42.6 ± 1.6	42.0 ± 1.1
Exposed	63.1 ± 3.5	52.0 ± 2.3*	50.7 ± 3.7*	51.3 ± 2.1*	43.8 ± 1.4	51.5 ± 1.8*	48.3 ± 1.6
MCHC (l/l)							
Control	0.21 ± 0.03	0.18 ± 0.01	0.18 ± 0.00	0.18 ± 0.01	0.19 ± 0.01	0.20 ± 0.00	0.20 ± 0.00
Exposed	0.21 ± 0.01	0.18 ± 0.00	0.17 ± 0.01	0.18 ± 0.01	0.18 ± 0.00	0.20 ± 0.00	0.19 ± 0.00

Erythrocyte count (RBC), hematocrit value (PCV), hemoglobin concentration (Hb), leukocyte count (WBC), mean erythrocyte volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC)

Note: Data are presented as mean ± SEM; an asterisk corresponds to significant differences between the exposed and corresponding control group, $^*P < 0.05$

with the control fish. The control females uniformly exhibited primary oocytes only, but in exposed fish scattered oocytes in the vitellogenic stage were visible.

Additionally, the structure of the ovaries in exposed females was more irregular compared with that of the controls, showing interstitial edema, higher amounts of

Table 4 Effect of STP effluent on biochemical blood plasma parameters in fish from control and Cezarka pond at 30 days

Biochemical blood parameters at 30 days for control and exposed fish		
Parameters	Control	Exposed
ALB (g/l)	7.0 ± 0.7	8.2 ± 0.3
ALT (U/l)	36.8 ± 8.6	26.5 ± 3.3
AST (U/l)	125 ± 13	188 ± 25*
Ca^{2+} (mmol/l)	2.66 ± 0.07	2.78 ± 0.04
LDH (u/l)	2277 ± 399	4826 ± 1263*
NH_3 (mmol/l)	609 ± 49	704 ± 59
CREA (mmol/l)	40.6 ± 6.9	78.8 ± 8.6*
TP (g/l)	22.4 ± 1.7	26.1 ± 1.3
PHOS (mmol/l)	5.2 ± 0.4	5.0 ± 0.2
Mg (mmol/l)	1.9 ± 0.1	2.0 ± 0.1
TRIG (mmol/l)	5.0 ± 0.8	2.0 ± 0.2*
LAC (mmol/l)	9.8 ± 0.6	16.1 ± 0.7
GLU (mmol/l)	4.18 ± 0.57	2.72 ± 0.33*
CK (U/l)	1173 ± 288	2212 ± 1016
ALP (U/l)	13.3 ± 1.3	8.8 ± 0.7*

Glucose (GLU), total proteins (TP), albumins (ALB), ammonia (NH_3), triglycerides (TRIG), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatine kinase (CK), lactate (LACT), alkaline phosphatase (ALP), calcium (Ca^{2+}), magnesium (Mg), and inorganic phosphate (PHOS)

Note: Data are presented as mean ± SEM; $n = 12$; an asterisk corresponds to significant differences between the exposed and corresponding control group, $*P < 0.05$

atretic oocytes, and inflammation with mainly macrophages and lymphocytes (Fig. 3). The pathology observations in the liver and gills were not significantly different between the control and exposed fish. However, in three Cezarka-exposed individuals, there was moderate infiltration with lymphocytes and macrophages (mainly perivascular) in the liver. No histopathology changes were evident in the kidneys or spleens.

Vitellogenin

Results of plasma VTG protein concentrations and relative hepatic mRNA expression exhibited the same trends (Fig. 4). The level of plasma VTG was generally higher in males and females from Cezarka pond. However, significant differences were registered in only females after 180 days and in both males and females after 360 days of exposure. The same results were observed in mRNA expression level in the liver. However, due to large fluctuation in the hepatic relative mRNA expression between individuals, the values were not significantly different in males. The highest levels of plasma VTG in males and females were 9.2 and 5600 µg/ml in exposed fish, respectively. The lowest level observed in control fish was 0 µg/ml for both sexes at 360 days.

Hematological Parameters and Biochemical Blood Plasma Parameters

The hematological properties of common carp in the Cezarka pond are shown in Table 3. The levels of Hb and RBC in effluent-exposed fish were significantly lower than in control fish after 90 days (by 13 and 16%, respectively). The numbers of WBC constantly decreased in the exposed group on day 90 (55), 180 (61), and 360 (74%) compared with the control fish. At 180 days of exposure, the levels of MCH in the exposed fish were significantly higher than in the control fish (by 27%). MCV were significantly higher in the exposed animals after 180 and 360 days of exposure. Although both male and female-exposed fish had higher levels of MCV, the elevations were significant in only females at 180 days and males at 360 days.

The plasma biochemical parameters of the carp exposed to STP discharges were investigated at day 30 only, and the results are shown in Table 4. After 30 days, AST, LDH, and CREA levels were significantly higher (by 1.5-fold, 2.1-fold, and 1.9-fold, respectively) in STP effluent-exposed fish. In contrast, the TRIG, GLU, and ALP concentrations were significantly lower (by 2.5-fold, 1.5-fold, and 1.5-fold, respectively) in STP effluent-exposed fish compared with the control fish. No significant changes in ALB, ALT, Ca, NH_3, TP, PHOS, Mg, LAC, or CK levels were found in the blood plasma of the fish.

Integrated Biomarker Response

The results of IBR and star plots are presented in Fig. 5. The IBR was calculated according to the time points that include the score of each parameter in each tissue. A IBR indicates to a high response. The IBR of exposed fish was much higher than in the control fish in the liver at 30 days (3.15 and 1.57, respectively) and in the intestine at 360 days (3.90 and 2.04). Additionally, the IBR was higher in the gills of exposed fish at 90 days and 180 days and in the livers at 360 days compared with the control fish. The star plots show the response of each biomarker for each tissue and time point (Fig. 5a). Different responses were observed for all of the biomarkers in the liver at 30 days. Furthermore, high responses were noted for GST and GR in the liver at 90 days, as well as SOD, GPx, and CAT in the intestine at 360 days.

Discussion

Occurrence of PPCPs

The highest concentration of PPCPs was detected in spring (30 days of exposure in May), and the lowest was detected

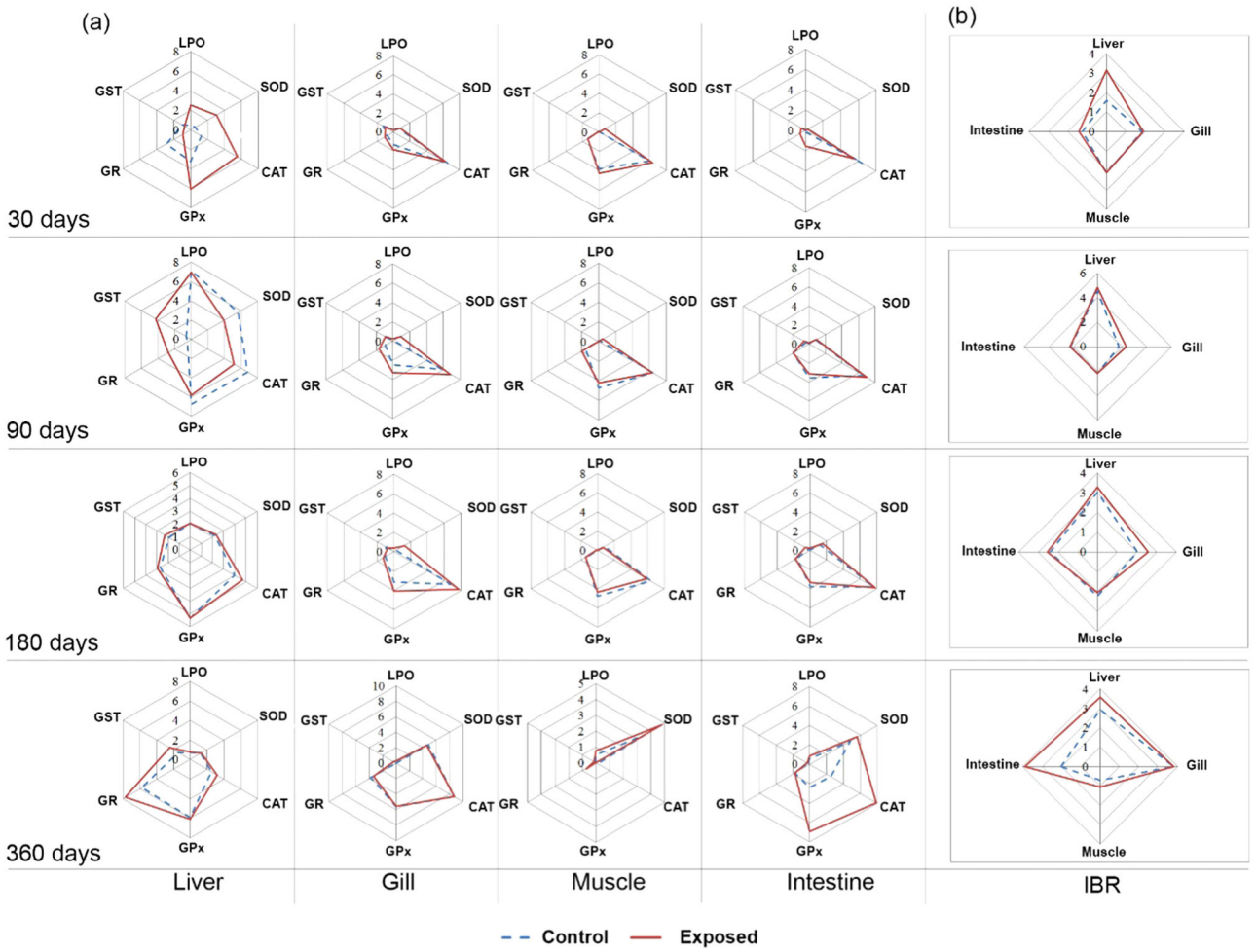

Fig. 5 Star plot for each sampling time (30, 90, 180, and 360 days) in each tissue (liver, gill, intestine, muscle) (**a**); and integrated biomarker response of all six biomarkers, including glutathione S-tranferase (GST), lipid peroxidation (LPO), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) at each sampling time point (**b**)

in autumn (180 days of exposure). The PPCP concentrations measured in Cezarka pond, which receives water from only STP effluent, differed by one to two orders of magnitude compared with the control pond, which receives surface water from the Blanice River. These results are in agreement with literature findings that STPs are a major source of PPCP pollutants in aquatic environments (Zheng and Li 2013).

Irbesartan, telmisartan, tramadol, carbamazepine, and its metabolite trans-dihydro-dixydroxy carbamazepine were the dominant contaminating compounds in Cezarka pond. The variation in PPCP concentrations can be attributed to increased human consumption of PPCPs during winter and spring. Furthermore, the low temperature during this period often results in less efficient removal of compounds during STP treatment (Golovko et al. 2014). Our results are consistent with previous findings of high PPCP concentrations detected in winter compared with summer (Golovko et al. 2014; Koba et al. 2017). Unfortunately, the control pond was not absolutely free of PPCPs due to the practically ubiquitous contamination of surface water by pharmaceutically active substances. However, the total level of PPCPs was low and can be referred to as a background concentration under central European conditions (Ebele et al. 2017).

Morphological Indices

Fish living in polluted environments are believed to reduce their CF because they have to expend energy for detoxification (Fang et al. 2009). It has been demonstrated that the CF declines in fish exposed to environmental pollutants (Khan 2003; Roussel et al. 2007). Fish exposed to STP effluents in Cezarka pond had higher CFs than those from the control pond. This can be explained by the abundance of STP-related nutrients in Cezarka pond compared with the control pond (Supplementary Material 3), resulting in highly fertile conditions with increased phytoplankton

productivity serving as dietary components for fish. This result is consistent with previous studies indicating that STP led to increased water nutrient loads (Björn Gücker 2006) and CF (Tetreault et al. 2013) for downstream fish.

In contrast to Cezarka, the trophic conditions in the control pond were representative of average aquaculture ponds, with an average natural weight gain of 0.5 kg (without artificial feeding) per season for different sizes of carp. Extreme growth intensity in Cezarka pond led to a high total fish biomass at 90 days. High feeding pressure in this period caused a significant reduction of available natural food. In the following period, high competition for food resulted in a dramatic decrease of CF, reaching comparable levels to the control pond at 180 days. The effect of STP effluent on CF was not different between sexes (Table 1).

The HSI reflects the relative liver size and is linked to the hepatic enzyme activity for the detoxification of pollutant compounds (Li et al. 2010; Yeom et al. 2007). An elevation of HSI was observed in carp that had been captured in a metal-contaminated site (Bervoets et al. 2009; Ozmen et al. 2006). Other studies found elevated HSI values in fish from sites contaminated with PCBs and PAHs compared to fish from uncontaminated sites (Pinkney et al. 2001). However, several natural factors might significantly affect the HSI of fish as well, such as nutrient levels and feeding strategies (Turano et al. 2007).

Significantly lower HSIs were observed in the exposed group with an exception of the results at 90 days. The reduction in liver size is likely linked to glycogen depletion due to the fish expending energy for detoxification activities. This assumption is supported by the low level of GLU in the blood plasma of the fish. The results of plasma biochemical parameters (AST, ALP), oxidative stress, and antioxidant enzymes (all parameters) might suggest damage to the liver tissue at 30 days. The histopathological examination after 360 days of exposure revealed a slight but non-significant decrease in the amount of glycogen vacuoles in hepatocytes in exposed fish compared to controls. The higher HSI in exposed fish compared with controls at 90 days might be explained by the enormous excess of natural food, which allow for deposition of excess energy in the liver. This result is consistent with the highest CF value observed at 90 days in fish from Cezarka pond. The same trend of HSI was observed in both male and female fish from Cezarka pond (Table 1).

The GSI has been used as a biomarker in aquatic organisms for exposure to environmental estrogens. Correlations have been established between the inhibition of testicular growth and the potency of estrogenic compounds in male fish (Gimeno et al. 1997). Field studies have reported that estrogenic chemicals decrease the GSI of exposed fish (Kukkonen et al. 1999). The GSI was lower among fish exposed to river water with high concentrations of estrogenic compounds, such as nonylphenol, bisphenol A, and 17β-estradiol (Hassanin et al. 2002). However, in this study, GSIs were higher in exposed female (at 180 and 360 days) and male fish (at 360 days) from Cezarka pond than in control fish (Table 1).

These results might indicate that the nutrient-rich environment in Cezarka pond forced exposed fish to grow larger gonads or to reproduce earlier compared to fish in the control pond. To confirm this hypothesis, fish gonad histology, VTG expression, and its concentration level were assessed (see section "Gonad histology and VTG biomarkers"). The decrease of GSI in control fish at 360 days can be explained by the expense of energy for the winter period. In Cezarka pond, warmer water from the STP effluent helped the fish to grow. The same trend of GSI was observed between male and female fish from Cezarka pond.

Gonad Histology and VTG Biomarkers

Previous studies have demonstrated an increase in VTG concentration in female common carp in streams receiving effluent water from STPs (Petrovic et al. 2002). In the present study, the gonads of exposed females were more developed compared with fish in the control group. VTG levels of females in the effluent-exposed group were higher at days 180 and 360 than those in the control group. The highly elevated level of VTG in exposed female fish and irregular ovary structures suggest a synergistic effect of xenoestrogens (Rankouhi et al. 2002). In addition, the high concentration of VTG in males is definitely linked to the action of the PPCP mixture with eventual estrogenic action, even if there were no structural changes visible in male gonads. Early gonad maturation of fish in the pond receiving water from the STP might lead to changes in the spawning success. Additional studies are necessary to examine regarding the reproduction success of fish under long-term exposure to a biological pond receiving only STP effluent.

Oxidative Stress and Antioxidant Enzyme Biomarkers

Considering the trend of each biomarker for all tissues and sampling time points, the effluent-exposed fish showed a general elevation of TBARs and SOD and a decline in CAT, GPx, and GR compared with control fish. SOD is known to be the first line of defense in response to the conversion of superoxide anion radicals to molecular oxygen and hydrogen peroxide (Fridovich 1989). The following steps are completed by other enzymes, such as CAT, GPx, and GR. The decline of CAT, GPx, and GR indicates that the fish's abilities to protect cells from hydrogen peroxide were reduced. The inhibition of CAT, GPx, and GR was reported in fish exposed to carbamazepine (Li et al.

2009), resulting in increased H_2O_2 in the cells (Ahmad et al. 2000). This result is consistent with hypotheses that elevated SOD activities can be combined with a decrease in CAT and GPx (Huang et al. 2007; Pandey et al. 2003; Stanic et al. 2006).

Different intensities of oxidative stress and antioxidant enzymes for different tissues and exposure periods have been indicated. The largest amount of PPCPs detected at 30 days obviously induced changes in oxidative stress and the system responses of antioxidant enzymes in the fish livers. The liver was the most responsive tissue in this period, and significant differences were noted between the control and effluent-exposed fish in all of the measured biomarkers.

At 180 days, most effects were evident in the gill tissue, which followed the same trend as the liver (an elevation in SOD and a decline in CAT, GPx, and GR). Interestingly, significant changes in SOD, CAT, GPx, and GR antioxidant enzymes did not lead to oxidative damage investigated according to TBAR levels. The opposite situation occurred at day 360 with increased TBARs but no respective alteration of enzyme activities in the gills. One potential explanation for this finding is that the antioxidant system of fish reacts to early exposure, and then oxidative damage follows. After 180 days of exposure, antioxidant enzymes were activated, but no damage occurred after the activation. In a later period, while the antioxidant system stabilized, oxidative damage caused by previous disturbances was evident.

Fewer changes were observed in intestine and muscle tissues. A significant reduction in the GPx level was detected at 360 days of exposure in combination with increasing CAT, which may be related to the damage induced by a high density of parasites (tapeworm—*Caryophyllaeus* sp.) detected in the intestines in this period (Supplementary Material 6). The decrease of GPx level has been observed in intestinal parasitic infections in humans (Mahittikorn et al. 2014).

Phase II Detoxification Enzyme (GST) in Liver, Gill, Intestine, and Muscle

The liver was the most responsive tissue in terms of GST activity. Considering the changes in GST for all tissues and sampling times, in most cases, high levels of GST were observed in exposed fish compared with the control fish. This result is consistent with previous studies of goldfish (Kubrak et al. 2012) and common carp (Schmidt et al. 2004) exposed to cobalt and polychlorinated biphenyl. Interestingly, low levels of GST were observed in the exposed fish livers at 30 days of exposure compared with the control fish. The result suggests an inhibition of GST due to the highest concentration of PPCPs detected in this period. A previous study also detected the inhibition of GST in the liver of common carp exposed to the herbicide quinclorac (Cavalheiro de Menezes et al. 2012). The lack of differences observed at day 180 can be explained by the lowest concentration of PPCPs detected in Cezarka pond at this time point.

Hematological Parameters and Biochemical Blood Plasma Parameters

The same trend was observed between male and female fish for most of changes in hematological parameters in Cezarka pond. The reduction of Hb and RBC at only day 90 could be linked with the variation of total PPCPs, which was higher in this period compared with later periods in Cezarka and the control pond. Unfortunately, hematological parameters at day 30 were not investigated due to the limited amount of blood samples of juvenile fish. Several studies have described a decrease in RBC and Hb in carp (Sudova et al. 2009), rainbow trout (Li et al. 2011a), and striped catfish (*Mystus vittatus*) (John 2007) exposed to contaminated environments.

Decreases in Hb concentration and RBC count levels are linked to anemia (Li et al. 2011a). Changes in WBC are recognized as a sensitive indicator of environmental stress (Cole et al. 2001). Declines in WBC detected at 90, 180, and 360 days reflect the constant disturbance of the immune system. Additionally, the increases in MCV and MCH (180 days) and PCV and MCV (360 days) suggest changes in the internal equilibrium of exposed fish. The effect of 62 PPCPs occurring in water may induce both a synergistic effect and an antagonistic effect. Therefore, the variation of MCV and MCH may not exactly reflect the trend of the total PPCP concentration in each sampling event, which was higher at 90 days. These changes may be caused by other factors, such as the occurrence of parasites detected at 360 days.

Due to numerous significant changes detected at day 30, the biochemical parameters in blood plasma were additionally investigated. The increase in AST and decrease in ALP may be linked to alterations in the liver. The increase in AST activity in plasma may be due to liver damage, which results in the liberation of intercellular enzymes and elevated plasma aminotransferase levels (Rao 2006b). Elevated AST has been observed in common carp exposed to trifluralin (Poleksić and Karan 1999) and deltamethrin (Velíšek et al. 2006) and was proposed as a biomarker of acute hepatic damage (Abdel-Tawwab et al. 2013).

ALP is produced by cells lining the small bile ducts in the liver. The decline in ALP may be linked to a decrease of this function in the liver. Previous studies have indicated a decrease in ALP level in the blood plasma of common carp exposed to chemical stress conditions (Dobsikova et al.

2006). In addition, the increasing level of LDH in the present study could indicate tissue damage, hypoxic conditions, and a switch to anaerobic metabolism (Nemcsok and Benedeczky 1990; Saravanan et al. 2011).

LDH is a tetramer of anaerobic glycoses. It is crucial for muscle physiology, particularly under conditions of chemical stress when a high level of energy may be required over short periods of time (Monteiro et al. 2007). Other changes suggest disturbances in kidney function (CRE) and energy metabolism (GLU, TRIG). Creatine is mainly removed from the blood by the kidneys. Thus, increased CRE levels indicate that pollution affects kidney function (Abdel-Tawwab et al. 2013). Decreases in GLU and TRIG were also observed in common carp exposed to antimicrobial peptide (Dong et al. 2015).

Integrated Biomarker Response

The IBR index is often used to describe general effects and to assess ecological risk by combining the results of a wide set of biomarkers (Beliaeff and Burgeot 2002). The usage and efficiency of this index have been demonstrated in many studies (Ferreira et al. 2015; Li et al. 2011b). In the present study, the IBR values clearly reflected a high response in the liver during the first period of exposure, when fish were small (early stage) and sensitive to the polluted environment. The liver is the most important organ for metabolizing PPCPs.

It is obvious that high PPCP concentrations in Cezarka pond affected the response of the fish livers. After this period, a small effect was observed on the gills. The adaptation of fish can be explained by warm weather periods (summer and autumn), when the growth conditions of fish were optimal and there were seasonally low PPCPs in Cezarka pond. A strong response was noted in the intestine at 360 days of exposure. This finding could be related to the high density of parasites detected in the intestine.

Conclusion

Our data have demonstrated that the biological pond Cezarka contained significantly higher PPCP concentrations compared with the control pond. Seasonal variations of PPCPs were observed, with the highest concentrations occurring in spring. Several effects of the treated effluent on exposed fish were observed in this scenario. The greatest effects were found in fish in the early stages of exposure, with rapid changes observed in numerous parameters. The observations of oxidative stress, antioxidant enzymes, and biochemical blood plasma parameters might indicate alterations in liver metabolism. The initial rapid response to the effluent environment was followed by a weakening biomarker response, which corresponds to a decrease of PPCP load. However, indications of endocrine disruption and oxidative stress were noted at later stages.

Despite the numerous physiological alterations observed in exposed fish, the fish growth was greater in the exposed pond than the control pond. The effects of pollutants and consequent physiological alterations in fish organs were probably compensated by the high nutrient content with a high availability of natural food. At the end of the experiment, the exposed fish were in good condition. However, their reproduction ability remains to be tested.

Although modest negative effects were observed in fish after long-term exposure in the STP effluent reservoir, the impact of the effluent after retention in the biological pond cannot be ignored in the receiving ecosystem. The effects of STP effluent on natural water bodies are highly dependent on the dilution factor, which must be considered in low-flow receiving streams. To minimize the impact, longer retention of water in biological ponds and balanced microbial, macrophyte, plankton, and fish communities must be maintained to achieve maximum degradation and removal capacity.

Acknowledgements The study was financially supported by the Ministry of Education, Youth and Sports of the Czech Republic—projects "CENAKVA" (No. CZ.1.05/2.1.00/01.0024), "CENAKVA Center Development" (No. CZ.1.05/2.1.00/19.0380), "CENAKVA II" (No. LO1205 under the NPU I program), by the Grant Agency of the University of South Bohemia in Ceske Budejovice (No. 012/2016/Z) and by the Czech Science Foundation (No. GACR 15-04258S). The authors would also like to thank the Vodnany town and CEVAK a.s. for supporting this work.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

References

Abdel-Tawwab M, Mousaad MN, Sharafeldin KM, Ismaiel NE (2013) Changes in growth and biochemical status of common carp, *Cyprinus carpio* L. exposed to water-born zinc toxicity for different periods. Int Aquat Res 5:11. https://doi.org/10.1186/2008-6970-5-11

Ahmad I, Hamid T, Fatima M, Chand HS, Jain SK, Athar M, Raisuddin S (2000) Induction of hepatic antioxidants in freshwater catfish (*Channa punctatus* Bloch) is a biomarker of paper mill effluent exposure. Biochim Biophys Acta 1:37–48

Anway MD, Cupp AS, Uzumcu M, Skinner MK (2005) Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science 308:1466–1469

Beliaeff B, Burgeot T (2002) Integrated biomarker response: a useful tool for ecological risk assessment. Environ Toxicol Chem 21:1316–1322

Bervoets L, Van Campenhout K, Reynders H, Knapen D, Covaci A, Blust R (2009) Bioaccumulation of micropollutants and

biomarker responses in caged carp (*Cyprinus carpio*). Ecotoxicol Environ Saf 72:720–728. https://doi.org/10.1016/j.ecoenv.2008.10.008

Björn Gücker MB, Martin T, Pusch (2006) Effects of wastewater treatment plant discharge on ecosystem structure and function of lowland streams. J North Am Benthol Soc 25(2):313–329

Bostock J, Lane A, Hough C, Yamamoto K (2016) An assessment of the economic contribution of EU aquaculture production and the influence of policies for its sustainable development. Aquac Int 24:699–733. https://doi.org/10.1007/s10499-016-9992-1

Brandao FP, Rodrigues S, Castro BB, Goncalves F, Antunes SC, Nunes B (2013) Short-term effects of neuroactive pharmaceutical drugs on a fish species: biochemical and behavioural effects. Aquat Toxicol 145:218–229

Broeg K, Lehtonen KK (2006) Indices for the assessment of environmental pollution of the Baltic Sea coasts: integrated assessment of a multi-biomarker approach. Mar Pollut Bull 53:508–522

Calisto V, Esteves VI (2009) Psychiatric pharmaceuticals in the environment. Chemosphere 77:1257–1274. https://doi.org/10.1016/j.chemosphere.2009.09.021

Cavalheiro de Menezes C et al. (2012) The effects of diphenyl diselenide on oxidative stress biomarkers in *Cyprinus carpio* exposed to herbicide quinclorac (Facet(R)). Ecotoxicol Environ Saf 81:91–97

Claiborne A (1985) Catalase activity CRC handbook of methods for oxygen radical research. CRC Press, Boca Raton, 1:283–284

Cleuvers M (2003) Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. Toxicol Lett 142:185–194

Cole MB, Arnole DE, Watten BJ, Krise WF (2001) Haematological and physiological responses of brook charr, to untreated and limestone-neutralized acid mine drainage. J Fish Biol 59:79–91. https://doi.org/10.1111/j.1095-8649.2001.tb02339.x

Cribb AE, Leeder JS, Spielberg SP (1989) Use of a microplate reader in an assay of glutathione reductase using 5,5′-dithiobis(2-nitrobenzoic acid). Anal Biochem 183:195–196. https://doi.org/10.1016/0003-2697(89)90188-7

Dobsikova R, Velisek J, Wlasow T, Gomulka P, Svobodova Z, Novotny L (2006) Effects of cypermethrin on some haematological, biochemical and histopathological parameters of common carp (*Cyprinus carpio* L.). Neuro Endocrinol Lett 2:91–95

Dong X-Q, Zhang D-M, Chen Y-K, Wang Q-J, Yang Y-Y (2015) Effects of antimicrobial peptides (AMPs) on blood biochemical parameters, antioxidase activity, and immune function in the common carp (*Cyprinus carpio*). Fish Shellfish Immunol 47:429–434. https://doi.org/10.1016/j.fsi.2015.09.030

Ebele AJ, Abou-Elwafa Abdallah M, Harrad S (2017) Pharmaceuticals and personal care products (PPCPs) in the freshwater aquatic environment. Emerg Contam. https://doi.org/10.1016/j.emcon.2016.12.004

Eguchi K et al. (2004) Evaluation of antimicrobial agents for veterinary use in the ecotoxicity test using microalgae. Chemosphere 57:1733–1738

Fang JK, Au DW, Wu RS, Chan AK, Mok HO, Shin PK (2009) The use of physiological indices in rabbitfish *Siganus oramin* for monitoring of coastal pollution. Mar Pollut Bull 58:1229–1235

Fent K, Weston AA, Caminada D (2006) Ecotoxicology of human pharmaceuticals. Aquat Toxicol 76:122–159. https://doi.org/10.1016/j.aquatox.2005.09.009

Ferreira NG, Cardoso DN, Morgado R, Soares AM, Loureiro S (2015) Long-term exposure of the isopod *Porcellionides pruinosus* to nickel: costs in the energy budget and detoxification enzymes. Chemosphere 135:354–362

Frasco MF, Guilhermino L (2002) Effects of dimethoate and beta-naphthoflavone on selected biomarkers of *Poecilia reticulata*. Fish Physiol Biochem 26:149–156. https://doi.org/10.1023/a:1025457831923

Fridovich I (1989) Superoxide dismutases. An adaptation to a paramagnetic gas. J Biol Chem 264:7761–7764

Garcia-Reyero N, Lavelle CM, Escalon BL, Martinovic D, Kroll KJ, Sorensen PW, Denslow ND (2011) Behavioral and genomic impacts of a wastewater effluent on the fathead minnow. Aquat Toxicol 101:38–48

Gimeno S, Komen H, Venderbosch PWM, Bowmer T (1997) Disruption of sexual differentiation in genetic male common carp (*Cyprinus carpio*) exposed to an alkylphenol during different life stages. Environ Sci Technol 31:2884–2890. https://doi.org/10.1021/es970215h

Golovko O, Kumar V, Fedorova G, Randak T, Grabic R (2014) Seasonal changes in antibiotics, antidepressants/psychiatric drugs, antihistamines and lipid regulators in a wastewater treatment plant. Chemosphere 111:418–426. https://doi.org/10.1016/j.chemosphere.2014.03.132

Gonzalez-Gonzalez ED et al. (2014) Metals and nonsteroidal anti-inflammatory pharmaceuticals drugs present in water from Madin Reservoir (Mexico) induce oxidative stress in gill, blood, and muscle of common carp (*Cyprinus carpio*). Arch Environ Contam Toxicol 67:281–295

Grabic R, Fick J, Lindberg RH, Fedorova G, Tysklind M (2012) Multi-residue method for trace level determination of pharmaceuticals in environmental samples using liquid chromatography coupled to triple quadrupole mass spectrometry. Talanta 100:183–195. https://doi.org/10.1016/j.talanta.2012.08.032

Grabic R, Jurcikova J, Tomsejova S, Ocelka T, Halirova J, Hypr D, Kodes V (2010) Passive sampling methods for monitoring endocrine disruptors in the Svratka and Svitava rivers in the Czech Republic. Environ Toxicol Chem 29:550–555. https://doi.org/10.1002/etc.85

Gungordu A (2011) Evaluation of PAH metabolites in bile of common carp, *Cyprinus carpio* L. with fixed wavelength fluorescence in a field and laboratory study. Polycycl Aromat Compd 31:84–96. https://doi.org/10.1080/10406638.2011.572576

Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 249:7130–7139

Halling-Sørensen B, Nors Nielsen S, Lanzky PF, Ingerslev F, Holten Lützhøft HC, Jørgensen SE (1998) Occurrence, fate and effects of pharmaceutical substances in the environment—a review. Chemosphere 36:357–393. https://doi.org/10.1016/S0045-6535(97)00354-8

Hansen PD, Dizer H, Hock B, Marx A, Sherry J, McMaster M, Blaise C (1998) Vitellogenin—a biomarker for endocrine disruptors TrAC. Trends Anal Chem 17:448–451. https://doi.org/10.1016/S0165-9936(98)00020-X

Hassanin A, Kuwahara S, Nurhidayat, Tsukamoto Y, Ogawa K, Hiramatsu K, Sasaki F (2002) Gonadosomatic index and testis morphology of common carp (*Cyprinus carpio*) in rivers contaminated with estrogenic chemicals. J Vet Med Sci 64:921–926

He X et al. (2011) Assessment of typical pollutants in waterborne by combining active biomonitoring and integrated biomarkers response. Chemosphere 84:1422–1431

Howcroft CF, Amorim MJ, Gravato C, Guilhermino L, Soares AM (2009) Effects of natural and chemical stressors on *Enchytraeus albidus*: can oxidative stress parameters be used as fast screening tools for the assessment of different stress impacts in soils? Environ Int 35:318–324

Huang DJ, Zhang YM, Song G, Long J, Liu JH, Ji WH (2007) Contaminants-induced oxidative damage on the carp *Cyprinus carpio* collected from the upper Yellow River, China. Environ Monit Assess 128:483–488

Islas-Flores H, Gomez-Olivan LM, Galar-Martinez M, Colin-Cruz A, Neri-Cruz N, Garcia-Medina S (2013) Diclofenac-induced

oxidative stress in brain, liver, gill and blood of common carp (*Cyprinus carpio*). Ecotoxicol Environ Saf 92:32–38

John PJ (2007) Alteration of certain blood parameters of freshwater teleost *Mystus vittatus* after chronic exposure to Metasystox and Sevin. Fish Physiol Biochem 33:15–20. https://doi.org/10.1007/s10695-006-9112-7

Khan RA (2003) Health of flatfish from localities in Placentia Bay, Newfoundland, contaminated with petroleum and PCBs. Arch Environ Contam Toxicol 44:485–492

Kilty CG, Keenan J, Shaw M (2007) Histologically defined biomarkers in toxicology. Expert Opin Drug Saf 6:207–215

Koba O et al. (2017) Transport of pharmaceuticals and their metabolites between water and sediments as a further potential exposure for aquatic organisms. J Hazard Mater 342:401–407

Köck-Schulmeyer M, Ginebreda A, González S, Cortina JL, de Alda ML, Barceló D (2012) Analysis of the occurrence and risk assessment of polar pesticides in the Llobregat River Basin (NE Spain). Chemosphere 86:8–16. https://doi.org/10.1016/j.chemosphere.2011.08.034

Kortenkamp A, Backhaus T, Faust M (2009) State of the art report on mixture toxicity. Report to the European Commission

Kubrak OI, Rovenko BM, Husak VV, Vasylkiv OY, Storey KB, Storey JM, Lushchak VI (2012) Goldfish exposure to cobalt enhances hemoglobin level and triggers tissue-specific elevation of antioxidant defenses in gills, heart and spleen. Comp Biochem Physiol C Toxicol Pharmacol 155:325–332

Kukkonen JVK, Punta E, Koponen P, Paranko J, Leppänent H, Holopainen IJ, Hyvärinen H (1999) Biomarker responses by crucian carp (*Carassius Carassius*) living in a pond of secondary treated pulp mill effluent. Water Sci Technol 40:123–130. https://doi.org/10.1016/S0273-1223(99)00709-X

Li Z-H et al. (2011a) Chronic toxicity of verapamil on juvenile rainbow trout (*Oncorhynchus mykiss*): effects on morphological indices, hematological parameters and antioxidant responses. J Hazard Mater 185:870–880. https://doi.org/10.1016/j.jhazmat.2010.09.102

Li Z-H, Zlabek V, Velisek J, Grabic R, Machova J, Randak T (2009) Responses of antioxidant status and Na^+–K^+-ATPase activity in gill of rainbow trout, *Oncorhynchus mykiss*, chronically treated with carbamazepine. Chemosphere 77:1476–1481. https://doi.org/10.1016/j.chemosphere.2009.10.031

Li ZH, Zlabek V, Li P, Grabic R, Velisek J, Machova J, Randak T (2010) Biochemical and physiological responses in liver and muscle of rainbow trout after long-term exposure to propiconazole. Ecotoxicol Environ Saf 73:1391–1396

Li ZH et al. (2011b) Evaluating environmental impact of STPs situated on streams in the Czech Republic: an integrated approach to biomonitoring the aquatic environment. Water Res 45:1403–1413

Lopez-Serna R, Petrovic M, Barcelo D (2012) Occurrence and distribution of multi-class pharmaceuticals and their active metabolites and transformation products in the Ebro river basin (NE Spain). Sci Total Environ 440:280–289

Mahittikorn A, Prasertbun R, Mori H, Popruk S (2014) Antioxidant enzyme activity among orphans infected with intestinal parasites in Pathum Thani Province, Thailand. Southeast Asian J Trop Med Public Health 45:1252–1263

Mills LJ, Chichester C (2005) Review of evidence: are endocrine-disrupting chemicals in the aquatic environment impacting fish populations? Sci Total Environ 343:1–34. https://doi.org/10.1016/j.scitotenv.2004.12.070

Mohandas J, Marshall JJ, Duggin GG, Horvath JS, Tiller DJ (1984) Differential distribution of glutathione and glutathione-related enzymes in rabbit kidney. Possible implications in analgesic nephropathy. Biochem Pharmacol 33:1801–1807

Monteiro M, Quintaneiro C, Nogueira AJ, Morgado F, Soares AM, Guilhermino L (2007) Impact of chemical exposure on the fish *Pomatoschistus microps* Kroyer (1838) in estuaries of the Portuguese Northwest coast. Chemosphere 66:514–522

Nava-Álvarez R, Razo-Estrada AC, García-Medina S, Gómez-Olivan LM, Galar-Martínez M (2014) Oxidative stress induced by mixture of diclofenac and acetaminophen on common carp (*Cyprinus carpio*). Water Air Soil Pollut 225:1–9. https://doi.org/10.1007/s11270-014-1873-5

Nemcsok J, Benedeczky I (1990) Effect of sublethal concentrations of phenol on some enzyme activities and blood sugar level of carp (*Cyprinus carpio* L.). Environ Monit Assess 14:377–383

Nishikimi M, Appaji Rao N, Yagi K (1972) The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochem Biophys Res Commun 46:849–854. https://doi.org/10.1016/S0006-291X(72)80218-3

Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95:351–358

Ozmen M, Güngördü A, Kucukbay FZ, Güler RE (2006) Monitoring the effects of water pollution on *Cyprinus carpio* in Karakaya Dam Lake, Turkey. Ecotoxicology 15:157–169. https://doi.org/10.1007/s10646-005-0045-1

Pandey S, Parvez S, Sayeed I, Haque R, Bin-Hafeez B, Raisuddin S (2003) Biomarkers of oxidative stress: a comparative study of river Yamuna fish Wallago attu (Bl. & Schn.). Sci Total Environ 309:105–115

Paraso MG, Morales JK, Clavecillas AA, Lola MS (2017) Estrogenic effects in feral male common carp (*Cyprinus carpio*) from Laguna de Bay, Philippines. Bull Environ Contam Toxicol 98:638–642

Petrovic M, Sole M, Lopez de Alda MJ, Barcelo D (2002) Endocrine disruptors in sewage treatment plants, receiving river waters, and sediments: integration of chemical analysis and biological effects on feral carp. Environ Toxicol Chem 21:2146–2156

Pinkney AE, Harshbarger JC, May EB, Melancon MJ (2001) Tumor prevalence and biomarkers of exposure in brown bullheads (*Ameiurus nebulosus*) from the tidal Potomac River, USA, watershed. Environ Toxicol Chem 20:1196–1205

Poleksić V, Karan V (1999) Effects of trifluralin on carp: biochemical and histological evaluation. Ecotoxicol Environ Saf 43:213–221. https://doi.org/10.1006/eesa.1999.1790

Rankouhi TR, van Holsteijn I, Letcher R, Giesy JP, van den Berg M (2002) Effects of primary exposure to environmental and natural estrogens on vitellogenin production in carp (*Cyprinus carpio*) hepatocytes. Toxicol Sci 67:75–80. https://doi.org/10.1093/toxsci/67.1.75

Rao JV (2006a) Biochemical alterations in euryhaline fish, *Oreochromis mossambicus* exposed to sub-lethal concentrations of an organophosphorus insecticide, monocrotophos. Chemosphere 65:1814–1820. https://doi.org/10.1016/j.chemosphere.2006.04.015

Rao JV (2006b) Toxic effects of novel organophosphorus insecticide (RPR-V) on certain biochemical parameters of euryhaline fish, *Oreochromis mossambicus*. Pestic Biochem Physiol 86:78–84. https://doi.org/10.1016/j.pestbp.2006.01.008

Rasmussen MK, Zamaratskaia G, Ekstrand B (2011) Gender-related differences in cytochrome P450 in porcine liver—implication for activity, expression and inhibition by testicular steroids. Reprod Domest Anim 46:616–623

Rio DC, Ares M Jr., Hannon GJ, Nilsen TW (2010) Purification of RNA using trizol (TRI reagent). Cold Spring Harb Protoc 5(6). http://dx.doi.org/10.1101/pdb.prot5439

Roussel H, Joachim S, Lamothe S, Palluel O, Gauthier L, Bonzom JM (2007) A long-term copper exposure on freshwater ecosystem using lotic mesocosms: individual and population responses of three-spined sticklebacks (*Gasterosteus aculeatus*). Aquat Toxicol 82:272–280

Saravanan M, Karthika S, Malarvizhi A, Ramesh M (2011) Ecotoxicological impacts of clofibric acid and diclofenac in common carp (*Cyprinus carpio*) fingerlings: hematological, biochemical, ionoregulatory and enzymological responses. J Hazard Mater 195:188–194. https://doi.org/10.1016/j.jhazmat.2011.08.029

Schmidt K, Steinberg CE, Pflugmacher S, Staaks GB (2004) Xenobiotic substances such as PCB mixtures (Aroclor 1254) and TBT can influence swimming behavior and biotransformation activity (GST) of carp (*Cyprinus carpio*). Environ Toxicol 19:460–470

Schoenfuss HL, Levitt JT, Van der Kraak G, Sorensen PW (2002) Ten-week exposure to treated sewage discharge has relatively minor, variable effects on reproductive behavior and sperm production in goldfish. Environ Toxicol Chem 21:2185–2190

Stanic B, Andric N, Zoric S, Grubor-Lajsic G, Kovacevic R (2006) Assessing pollution in the Danube River near Novi Sad (Serbia) using several biomarkers in sterlet *(Acipenser ruthenus* L.*)*. Ecotoxicol Environ Saf 65:395–402. https://doi.org/10.1016/j.ecoenv.2005.08.005

Sudova E, Piackova V, Kroupova H, Pijacek M, Svobodova Z (2009) The effect of praziquantel applied per os on selected haematological and biochemical indices in common carp (*Cyprinus carpio L.*). Fish Physiol Biochem 35:599–605

Svobodova Z, Pravda D, Modra H (2012) Method of haematological examination of fish (in Czech). Research Institute of Fish Culture and Hydrobiology, Vodnany, Vol. 122, p 38

Thibaut R, Schnell S, Porte C (2006) The interference of pharmaceuticals with endogenous and xenobiotic metabolizing enzymes in carp liver: an in-vitro study. Environ Sci Technol 40:5154–5160

Tetreault GR, Brown CJ, Bennett CJ, Oakes KD, McMaster ME, Servos MR (2013) Fish community responses to multiple municipal wastewater inputs in a watershed. Integr Environ Assess Manag 9:456–468

Turano MJ, Borski RJ, Daniels HV (2007) Compensatory growth of pond-reared hybrid striped bass, *Morone chrysops* × *Morone saxatilis*, fingerlings. J World Aquac Soc 38:250–261. https://doi.org/10.1111/j.1749-7345.2007.00094.x

Van der Oost R, Beyer J, Vermeulen NP (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ Toxicol Pharmacol 13:57–149

Velíšek J, Dobšíková R, Svobodová Z, Modrá H, Lusková V (2006) Effect of deltamethrin on the biochemical profile of common carp (*Cyprinus carpio* L.). Bull Environ Contam Toxicol 76:992–998. https://doi.org/10.1007/s00128-006-1016-9

Velisek J, Stara A, Kolarova J, Svobodova Z (2011) Biochemical, physiological and morfological responses in common carp (*Cyprinus carpio* L.) after long-term exposure to terbutryn in real environmental concentration. Pestic Biochem Physiol 100:305–313. https://doi.org/10.1016/j.pestbp.2011.05.004

White A, Fletcher TC (1985) Seasonal-changes in serum glucose and condition of the plaice. Pleuronectes-platessa L. *J Fish Biol*. 26:755–764. http://dx.doi.org/10.1111/j.1095-8649.1985.tb04316.x

Witeska M, Wakulska M (2007) The effects of heavy metals on common carp white blood cells in vitro. Altern Lab Anim 35:87–92

Yeom DH, Lee SA, Kang GS, Seo J, Lee SK (2007) Stressor identification and health assessment of fish exposed to wastewater effluents in Miho Stream, South Korea. Chemosphere 67:2282–2292

Zenobio JE, Sanchez BC, Leet JK, Archuleta LC, Sepulveda MS (2015) Presence and effects of pharmaceutical and personal care products on the Baca National Wildlife Refuge, Colorado. Chemosphere 120:750–755

Zheng SK, Li XF (2013) Pharmaceuticals and personal care products (PPCPs) in the effluent of sewage treatment plants. Huan Jing Ke Xue 34:3316–3326

Zivna D et al. (2016) The effects of ciprofloxacin on early life stages of common carp (*Cyprinus carpio*). Environ Toxicol Chem 35:1733–1740. https://doi.org/10.1002/etc.3317