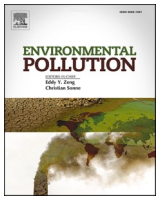
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Metabolome adaptation and oxidative stress response of common carp (*Cyprinus carpio*) to altered water pollution levels☆

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ABSTRACT

Treated wastewater ponds (TWPs) serve as recipients and passive tertiary treatment mediators for recycled water. These nutrient-rich habitats are increasingly utilised in aquaculture, nevertheless multiple loads of various contaminants with adverse effects on aquatic fauna, including fish, have been recorded. In the present study, we investigated the effects of fish transfer in response to altered levels of pollution on liver metabolic profiles and tissue-specific oxidative stress biomarkers during short- and long-term exposure. In a field experiment, common carp (*Cyprinus carpio*) originating in severely polluted TWP were restocked after one year to a reference pond with a background pollutant concentration typical of the regional river. In contrast, fish that originated in the reference pond were restocked to TWP. Fish were sampled 0, 7, 14, 60, and 180 days after restocking and fish liver, kidney, intestine, and gill tissues were subjected to biomarker analysis. Pharmaceutically active compounds (PhACs) and metabolic profiles were determined in fish liver using liquid chromatography high-resolution mass spectrometry (LC-HRMS). Fish transferred from reference to polluted pond increased the antioxidant response and absorbed PhACs into metabolism within seven days. Fish liver metabolic profiles were shifted rapidly, but after 180 days to a lesser extent than profiles in fish already adapted in polluted water. Restocked fish from polluted to reference pond eliminated PhACs during the short phase within 14 days, and the highest antioxidant response accompanied the depuration process. Numerous elevated metabolic compounds persisted in such exposed fish for at least 60 days. The period of two weeks was suggested as sufficient for PhACs depuration, but more than two months after restocking is needed for fish to stabilise their metabolism. This study contributed to determining the safe handling with marketed fish commonly restocked to wastewaters and clarified that water pollution irreversibly altered fish metabolic profile.

1. Introduction

Various chemical pollutants are released daily with municipal wastewater into wastewater treatment plants (WWTPs). A large group of micropollutants, defined as contaminants of emerging concern (CECs), persists in treated wastewater effluents (Rivera-Utrilla et al., 2013). The removal efficiency of CECs in WWTPs depends on the extent of treatment processes (physical, biological treatment, and advanced chemical

Abbreviations: CAT, catalase; CECs, contaminants of emerging concern; FCF, Fulton's condition factor; FFPW USB, Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione S-transferase; HSI, hepatosomatic index; IBRv2, integrated biomarker response version 2; LC-HRMS, liquid chromatography high-resolution mass spectrometry; LOQ, limit of quantification; LPO, lipid peroxidation; NSAIDs, nonsteroidal anti-inflammatory drugs; PCA, principal component analysis; PhACs, pharmaceutically active compounds; PMS, post-mitochondrial supernatant; POCIS, polar organic chemical integrative samplers; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TWP, treated wastewater pond; WWTP, wastewater treatment plant.

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oxidation), but their removal due to low concentrations and complex molecular structures is still inefficient (Golovko et al., 2014; Zhang et al., 2017). Recent studies have shown that subtle long-term exposure to CECs has no acute lethal effects but results in chronic risks and bioaccumulation in aquatic biota (Dobbins et al., 2009; Overturf et al., 2015). Typical representatives are pharmaceutically active compounds (PhACs) that are biologically active at low doses with consequences to nontarget organisms of aquatic biota (Buřič et al., 2018; Rezaei et al., 2020). It has been documented that environmentally relevant concentrations of PhACs affect biochemical and histological parameters in crayfish (Stara et al., 2019) and fish (Steinbach et al., 2016), feeding and reproductive behaviour of fish (Brodin et al., 2013), and aquatic insects and decapods (Bláha et al., 2019). In both laboratory and field exposure studies, PhACs have been detected in higher concentrations in fish liver, kidney, and brain compared to other tissues such as plasma and muscles (Grabicova et al., 2017; Grabicová et al., 2020; Lajeunesse et al., 2011).

Metabolomics and biological biomarker analysis are essential tools to assess a part of the internal exposome in the organism, where exposomes encompass organism life-course environmental exposures (Wild, 2012). An advanced instrumental metabolomic approach reveals adverse cellular responses and defense mechanisms to contaminant exposure and whole mechanisms of homeostasis in organisms. Determination of the metabolome in liver and blood plasma is a powerful tool for describing metabolic functional differences in fish exposed to effluent contaminants (Simmons et al., 2017). Endogenous metabolites are produced as substrates and intermediates participating in various common cellular processes. Their perturbation by contaminants could lead, for instance, to changes in energy and lipid metabolism in fish (Jordan et al., 2012). Targeted analysis using liquid chromatography high-resolution mass spectrometry (LC-HRMS) is a sensitive tool for quantifying specific compounds of interest in biological samples as animal tissues (Grabicova et al., 2018). Nontargeted metabolomics is a comprehensive approach for evaluating all metabolites, including unknown xenobiotic compounds in biological samples (Dunn et al., 2011). Xenobiotic compounds can be crutial triggers of reactive oxygen species (ROS) production during their metabolic transformation in organisms (Stoliar and Lushchak, 2012).

Reactive oxygen species, including radicals (superoxide anion, singlet oxygen, hydroxyl radical, and their derivatives) and nonradical hydrogen peroxide, occur naturally as byproducts in animal metabolic pathways (Lushchak, 2014). Oxidative stress in organisms can occur when the defense mechanisms participating in removing ROS are imbalanced with protective antioxidative enzymes and other nonenzymatic antioxidants (Lushchak, 2011). Lipid peroxidation (LPO) and the activities of antioxidative enzymes have been previously documented as biomarkers that can be used to monitor the level of secondary antioxidative stress induced by xenobiotics in both aquatic vertebrates and invertebrates (Cappello et al., 2016; Lidova et al., 2016; Stará et al., 2014). The integrative biomarker approach enables summarising and simplifying the interpretation of the multiple biomarker responses (Beliaeff and Burgeot, 2002).

Treated wastewater ponds (TWPs) are recipients of recycled water and are commonly used to decrease environmental pollution risks due to the passive elimination of micropollutants, particularly by photodegradation, biodegradation, and sorption (Fedorova et al., 2022). Recycled water is commonly used in small regional communities for various purposes, i.e., as a water source for aquaculture and irrigation of crops and parks and gardens (Nyanda and Mahonge, 2021). An experimental TWP with only one water input – an effluent from a regional WWTP – was selected in the present study. Fish cultured in ponds directly connected to TWP, especially in developing countries, are commonly consumed without any known direct health risks (Mara, 2001). Our previous studies showed that recycled water in TWP contained residual pharmaceuticals in concentrations up to μg L^{-1} in water (Fedorova et al., 2022; Giang et al., 2019). Previous experiments on fish stocked directly in TWP revealed bioaccumulation of PhACs in carp liver, kidney, and brain detectable in tens of ng g^{-1} wet tissue, whereas muscle showed only low levels of target analytes within the standards of consumption (Grabicová et al., 2020). We revealed shifted indicators of oxidative stress and histological changes in carp tissues when cultured in the same treated wastewater (Giang et al., 2019). Moreover, the micropollutants present in this experimental TWP induced hepatic and intestinal cytochrome P450-derived enzymes that affected carp fatty acid metabolism and the intestinal microbiome (Sakalli et al., 2018). Although the apparent alterations in metabolism in fish cultured during the whole season in polluted water have been documented and well studied, there is a lack of knowledge about metabolic adaptation processes after fish restocking to a higher or lower level of pollution. Both types of fish restocking reflect standard practices in pond aquaculture worldwide.

We hypothesised that fish metabolome depends on the water pollution level and fish metabolic profile is rapidly adaptable to the environment. The main aim was to determine time-dependent metabolic responses of fish restocked from polluted to clean water and in the opposite way and to compare with those of already adapted fish. We focused on determining targeted PhACs and nontargeted metabolic compounds, including unspecified xenobiotics and metabolites in fish liver. We aimed to assess lipid peroxidation and antioxidant enzyme activities in carp liver, kidney, intestine, and gill as fish stress and adaptation markers.

2. Materials and methods

2.1. Experimental location and sampling

Two experimental ponds were selected as representatives of different levels of pollution. Treated wastewater pond Čežárka (2.6 ha), a recipient of effluent from municipal WWTP (Vodňany town, 7000 inhabitants, South Bohemia, Czech Republic), was selected as the worst-case scenario. Standard technology in this WWTP includes primary physical filtration, sedimentation, biological oxidation, sludge sedimentation, and secondary settling processes. The Čežárka pond fed exclusively from the WWTP mediates a tertiary treatment process involving aquaculture. The reference pond (0.12 ha), which is connected to the Blanice River upstream of the town within the pond system of the Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice (FFWP USB), was selected as representative of a pond with a low background pollutant concentration typical of a regional river. The distance between the control pond and the TWP is approximately 2 km with comparable climatic conditions. The map of experimental ponds is available elsewhere (Giang et al., 2019).

One-year-old carp of the same genetic origin were used for the experiment. The first group of fish was reared in the first year of life at TWP Čežárka, and the second group was reared under the standard conditions of a reference pond in the aquaculture facilities of FFWP USB. The ponds were harvested in the spring of 2018. Individuals of similar size were selected from the fish groups mentioned above, group-marked, and stocked at defined densities back into the experimental ponds. Two groups, TWP→TWP and TWP→REF, were created from the fish cultured in TWP Čežárka (fish weight 50.8 ± 8.8 g, mean ± standard deviation). The TWP→TWP treatment group was released back into TWP Čežárka as a positive control, and the TWP→REF treatment group was restocked into the reference pond. Two groups, REF→REF and REF→TWP, were created from fish cultured under pond farming conditions of the FFWP USB (fish weight 48.0 ± 4.8 g). The REF→REF treatment group was added to the reference pond as a negative control, and the REF→TWP treatment group was restocked into TWP Čežárka. The ratio between stocked individuals in every two groups in both experimental ponds was approximately 1:1. Although both ponds differed in size, the total stocking density was similar (0.14 fish m^{-2}). Natural food was the main component of the stocks' nutrition in both experimental ponds. Common carp older than one year are mainly omnivorous bottom feeders of

benthic organisms, plant matter, and plankton in the diet in varying ratios (Anton-Pardo and Adámek, 2015).

Ten individual fish of each treatment and sampling time of each phase of exposure (start 0 days, short phase 7 and 14 days, and long phase 60 and 180 days) were caught by electrofishing. The sex ratio could not be reliably determined in juvenile fish, but males and females were sampled in an equal ratio at Day 180. Fish were sacrificed, and then tissues were taken and stored for future use (liver was stored at −20 °C for chemical analyses; liver, kidney, intestine, and gill were stored at −80 °C for biochemical analyses). Experimental animals were handled in accordance with the EU harmonized Animal Welfare Act of the Czech Republic. Our research unit is licenced (No. 53100/2013-MZE-17214) according to the Czech National Directive (the Law against Animal Cruelty, No. 246/1992). The experimental arrangement, sample collection and analysis are given in Fig. 1.

2.2. Water and sediment analyses

Water and sediment sampling was performed at selected fish sampling time points on Days 0, 60, and 180. Grab water samples were taken from a depth of 0.5 m below the water level at TWP Čežárka near the wastewater inlet and outflow from the pond and at the reference pond near the river water inlet. Water was filtered (0.20 μm, regenerated cellulose, Labicom) and frozen at −20 °C until analysis. Water PhACs were analysed using a method described by Lindberg et al. (2014). At the same sites, polar organic integrative samplers (POCIS) were installed 14 days before the regular sampling day according to a previously validated method (Fedorova et al., 2014). The analysis of POCIS was performed according to Vrana et al. (2021). Pond sediment was sampled using an Ekman-Birge sampler and frozen at −20 °C until analysis. Sediment samples were processed according to Koba et al. (2018). In addition, water temperature and pH were monitored, and basic hydrochemical parameters (i.e., ammonium, nitrates, nitrites, phosphates, chemical, and biological oxygen demand) were determined using standardised methods.

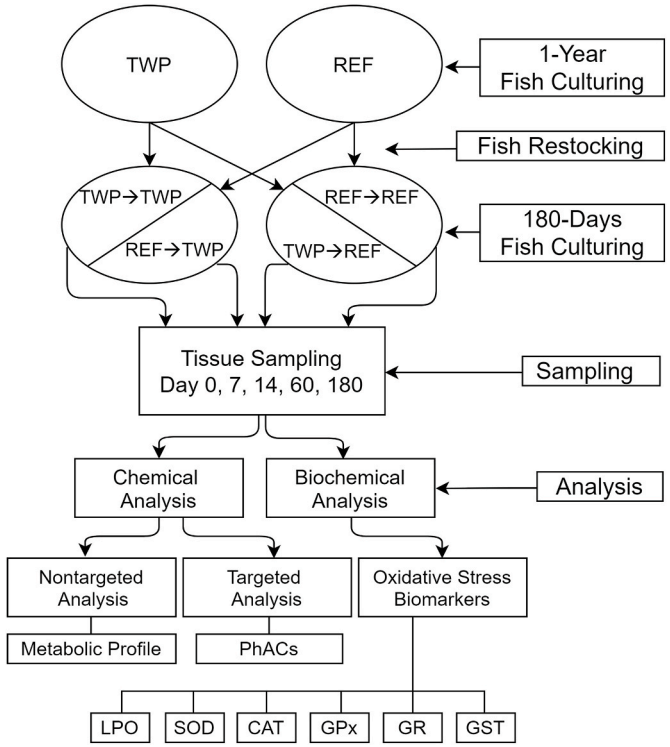


Fig. 1. Study design - experimental arrangement, sampling and analysis.

2.3. Fish morphological indices

The morphological indices of fish were determined using body weight and length and liver weight. Fulton's condition factor was calculated as FCF = (body weight [g]/total body length [cm]^3) x 100 (Robinson et al., 2008). The hepatosomatic index was evaluated as HSI = (liver weight [g]/body weight [g]) x 100 (White and Fletcher, 1985).

2.4. Chemical analyses of fish liver samples

For the chemical analyses, isotopically labeled internal standards, extraction solvent mixture, and homogenisation balls were added to 0.5 g of the thawed fish liver. The extraction was performed using a homogeniser (TissueLyser II, Quiagen, Germany, 1800 min^{-1} for 10 min) followed by centrifugation (10 000 rpm for 5 min; Mini spin, Eppendorf, Germany). The supernatant was filtered through a regenerated cellulose filter (0.45 μm, Labicom, Czech Republic) and stored at −20 °C for 24 h. The thawed supernatant was centrifuged again, and aliquots were placed in 2 mL vials and analysed by liquid chromatography high-resolution mass spectrometry (LC-HRMS, QExactive, Thermo Fisher Scientific, USA) operated in a high-resolution product scan. Details are given in Grabicova et al. (2018). The high-resolution product scan in positive mode was used to quantify selected PhACs, while a full scan in both positive and negative modes was used for the nontargeted analysis of the metabolome. The conditions and setup of instruments are listed in the supplementary material (SM1). The obtained quantification data were processed using TraceFinder 3.3, and full scan data were processed with Compound Discoverer 3.1 software (both Thermo Fisher Scientific, Inc., USA).

2.5. Biochemical analyses of fish tissue samples

Tissue homogenate and postmitochondrial supernatant (PMS) were prepared as described by Howcroft et al. (2009). An aliquot of homogenate was taken to determine the level of lipid peroxidation (LPO) expressed as thiobarbituric acid reactive substances (TBARS) according to Ohkawa et al. (1979). PMS aliquots were taken to assess antioxidant enzyme activities. Superoxide dismutase (SOD) activity was determined according to Ewing and Janero (1995) based on SOD-mediated inhibition of nitro blue tetrazolium reduction. Catalase (CAT) activity was detected according to the Claiborne (1985) protocol and modified to a 96-well UV microplate by Cappello et al. (2016). Glutathione peroxidase (GPx) activity was assessed according to Mohandas et al. (1984). Glutathione reductase (GR) activity was determined according to Cribb et al. (1989). Both GPx and GR activities were expressed as nicotinamide adenine dinucleotide phosphate oxidised min^{-1} mg^{-1} of protein. Glutathione S-transferase (GST) activity was assessed according to Habig et al. (1974) and expressed as 1-chloro-2,4-dinitrobenzene conjugate formed min^{-1} mg^{-1} of protein. Biochemical analyses are described in detail in the supplementary material (SM2).

2.6. Integrated biomarker response version 2 (IBRv2)

The multicomponent biomarker approach requires a method to summarise and simplify the complex results. An evaluation method, IBRv2 presented by Sanchez et al. (2013), was used in this work to determine the inductive or inhibitory effect of individual biomarkers.

2.7. Statistical analyses

One-way ANOVA, followed by Tukey's HST test, was applied to evaluate FCF and HSI and oxidative stress biomarkers after previous logarithmic transformation and Shapiro–Wilk's W test for data normality and Leven's test for homoscedasticity. Nonparametric Kruskal–Wallis ANOVA was applied if the assumptions were not satisfied. Means were considered significantly different at $P \leq 0.05$. The analyses

were performed using STATISTICA v. 12 (TIBCO Software Inc.).

Principal component analysis (PCA) was performed to evaluate targeted analytes of PhACs in fish liver using Canoco 5, Windows release, 5.10 version (Biometris, the Netherlands, and Petr Šmilauer, Czech Republic). Data were logarithmically transformed, and the concentrations of PhACs were applied as response variables. The concentrations were calculated from results above the limit of quantification (LOQ).

Differential analysis using $P \leq 0.001$ and $\log_2 = 1$ (threshold at least one order of magnitude difference in intensity) was applied to detect elevated and lowered compounds in nontargeted analysis in carp liver. PCA was applied to differentiate fish groups using scores of elevated and lowered compounds after previous data transformation. The analyses of the metabolic profile were performed using Compound Discoverer 3.1 software (Thermo Fisher Scientific, Inc., USA). The sample regression coefficients obtained in metabolomic PCA were applied to test correlations with FCF and HSI of each fish in each sampling group using a partial correlation test in STATISTICA v. 12 (TIBCO Software Inc.).

3. Results

3.1. Hydrochemical parameters and PhACs in water and sediment

Basic water characteristics were assessed in each of three seasons (spring, summer, and autumn) at fish sampling time points on Days 0, 60, and 180. The pH did not differ between the reference pond and TWP and ranged from 6.8 to 7.1. All hydrochemical parameters were elevated in the TWP compared to the reference pond (Table 1). Total nitrogen was on average 3–6 times higher, and total phosphorus was 25 times higher in the TWP than in the reference pond. Chemical and biological oxygen demand were on average up to 1.8 times and up to 3.1 times higher in TWP than in the reference pond, respectively. The total PhAC concentration in the grab water samples and POCIS was one order of magnitude higher in the TWP than in the reference pond (Table 1). Pharmaceutical concentrations that accumulated in the TWP sediment showed higher variability between the inlet and outflow and reached 10–65 times higher values than those in the sediment of the reference pond.

3.2. Fish sampling and morphological indices

Ten fish individuals of each sampling time and treatment were captured except for TWP→TWP on Day 60 and TWP→REF on Day 14, when only seven individuals were sampled. The dispersion of fish in TWP disabled catching any fish specimen from treatment TWP→TWP on Days 7 and 14. Despite similar initial weights in exposed and control fish, the FCF and HSI were significantly lower in the reference group. Fish from both exposed groups, TWP→TWP and REF→TWP, on Day 14 and Day 60 had significantly higher weight and HSI than fish groups REF→REF and TWP→REF living in the reference pond (Table 2). The final weights assessed on Day 180 did not differ among treatments, but

Table 2
Morphological parameters: total fish weight, Fulton's condition factor, and hepatosomatic index in common carp calculated for each sampling time and treatment. TWP→TWP – exposed fish to treated wastewater pond, REF→TWP – transferred fish from reference pond to treated wastewater pond, REF→REF – fish adapted to reference pond, and TWP→REF – fish transferred from treated wastewater pond to reference pond. Data represent the mean ± SD, n = 10, n = 7 in TWP→TWP Day 60 and TWP→REF Day 14; NS – not sampled.

	Day 0	Day 7	Day 14	Day 60	Day 180
Total fish weight (g)					
TWP→TWP	50.8 ± 8.8^{Aa}	NS	NS	385.7 ± 59.8^{Cb}	677.5 ± 51.2^{Ac}
REF→TWP	48.0 ± 4.8^{Aa}	45.0 ± 8.2^{Aa}	51.0 ± 10.2^{Ba}	301.5 ± 41.2^{Bb}	661.0 ± 91.2^{Ac}
REF→REF	48.0 ± 4.8^{Aa}	39.5 ± 5.5^{Aa}	33.7 ± 6.4^{Aa}	215.5 ± 31.9^{Ab}	616.5 ± 138.9^{Ac}
TWP→REF	50.8 ± 8.8^{Aa}	43.0 ± 8.6^{Aa}	35.0 ± 11.2^{Aa}	179.0 ± 30.4^{Ab}	557.5 ± 102.8^{Ac}
Fulton's condition factor					
TWP→TWP	2.01 ± 0.15^{Ba}	NS	NS	2.03 ± 0.15^{Aa}	1.47 ± 0.12^{Aa}
REF→TWP	1.64 ± 0.16^{Aab}	1.92 ± 0.08^{Bb}	1.69 ± 0.09^{Aab}	1.96 ± 0.23^{Ab}	1.50 ± 0.12^{Aa}
REF→REF	1.64 ± 0.16^{Aa}	1.65 ± 0.21^{Aab}	1.70 ± 0.35^{Aab}	1.91 ± 0.15^{Ab}	1.64 ± 0.12^{Bab}
TWP→REF	2.01 ± 0.15^{Bc}	1.80 ± 0.13^{ABb}	1.71 ± 0.14^{Aab}	1.91 ± 0.20^{Abc}	1.55 ± 0.12^{ABa}
Hepatosomatic index					
TWP→TWP	4.83 ± 0.75^{Bc}	NS	NS	3.39 ± 1.13^{Bb}	2.02 ± 0.23^{Aa}
REF→TWP	2.91 ± 0.77^{Ab}	3.38 ± 0.35^{Bb}	2.87 ± 0.29^{Ab}	3.32 ± 0.82^{Bb}	2.12 ± 0.26^{ACa}
REF→REF	2.91 ± 0.77^{Ab}	2.57 ± 0.51^{Aab}	3.53 ± 0.91^{Ab}	2.17 ± 0.32^{Aa}	2.59 ± 0.32^{Bab}
TWP→REF	4.83 ± 0.75^{Bb}	2.59 ± 0.44^{Aa}	2.57 ± 0.94^{Aa}	2.12 ± 0.31^{Aa}	2.37 ± 0.34^{BCa}

Means in a column followed by variable uppercase letters are significantly different among treatments at the given time; means in a row followed by variable lowercase letters are significantly different among sampling times in the same treatment ($P \leq 0.05\%$).

Table 1
Water temperature (Temp), hydrochemical properties, and sum of concentrations of pharmaceuticals and their transformation products (TPs) in water, polar organic chemical integrative samplers (POCIS), and sediment. Samples were taken from the inlet and outflow of the treated wastewater pond (TWP) and the inlet of the reference pond (REF). The data were assessed in one replicate.

	Day 0			Day 60			Day 180		
	TWP		REF	TWP		REF	TWP		REF
	Inlet	Outflow		Inlet	Outflow		Inlet	Outflow	
Temp (°C)	16.0	16.0	16.0	24.5	24.0	NA	16.5	10.5	9.9
Hydrochemical properties (mg L^{-1})									
NH_4^+-N	0.16	0.25	0.12	2.65	6.01	0.26	0.35	2.32	0.13
NO_3^--N	5.86	5.31	1.10	9.20	4.57	0.14	11.70	6.71	1.72
NO_2^--N	0.17	0.19	0.02	0.08	0.14	0.01	0.07	0.23	0.01
N_{tot}	9.1	8.5	2.6	12.1	10.9	1.8	12.6	10.7	2.0
PO_4^{3-}-P	0.25	0.02	0.01	0.30	0.63	0.24	0.12	0.64	0.03
P_{tot}	0.60	0.29	0.08	0.50	0.77	0.30	0.27	0.82	0.13
COD_{Mn}	20.2	19.5	11.2	11.8	12.2	9.9	12.2	20.5	13.8
COD_{Cr}	36.0	34.0	19.0	22.5	22.9	20.7	17.8	40.1	17.0
BOD_5	15.4	11.5	5.9	11.0	7.0	5.0	5.1	22.7	4.5
Sum of pharmaceuticals and TPs									
Water (ng L^{-1})	5900	5000	480	3400	4000	370	4300	4200	430
POCIS (ng $POCIS^{-1}$)	9700	7500	740	5100	3700	510	4300	2000	360
Sediment (ng g^{-1} dw)	120	320	5.0	580	120	9.0	300	220	10

significantly higher FCF and lower HSI were observed in the control REF→REF group.

3.3. Pharmaceutically active compounds in fish liver

Targeted analysis performed in fish liver detected 24 out of 77 tested PhACs above individual LOQs in at least one group at one sampling time. Fig. 2 shows the individual PhAC concentrations in carp liver at each treatment and sampling time. We included only concentrations identified in more than 50% of fish in one group.

The highest average concentrations of PhACs were quantified in exposed fish (TWP→TWP) on Day 0 and restocked fish to the TWP pond (REF→TWP) on Days 7 and 14, with average concentrations of 41, 31, and 30 ng g^{-1}, respectively. According to long exposure and fish body increments, the concentration of PhACs decreased up to only 6–9 ng g^{-1} on Days 60 and 180 from the experimental setup (Fig. 2A). A relative representation of individual therapeutic groups in TWP→TWP and TWP→REF liver samples showed that the concentrations of psychoactive drugs reached more than 75% of the total PhACs, which was similar to the ratio observed in sediment samples and more than 20% of cardiovascular drugs that were relatively abundant in water and POCIS (SM3). The PhACs with the highest concentrations in the liver were

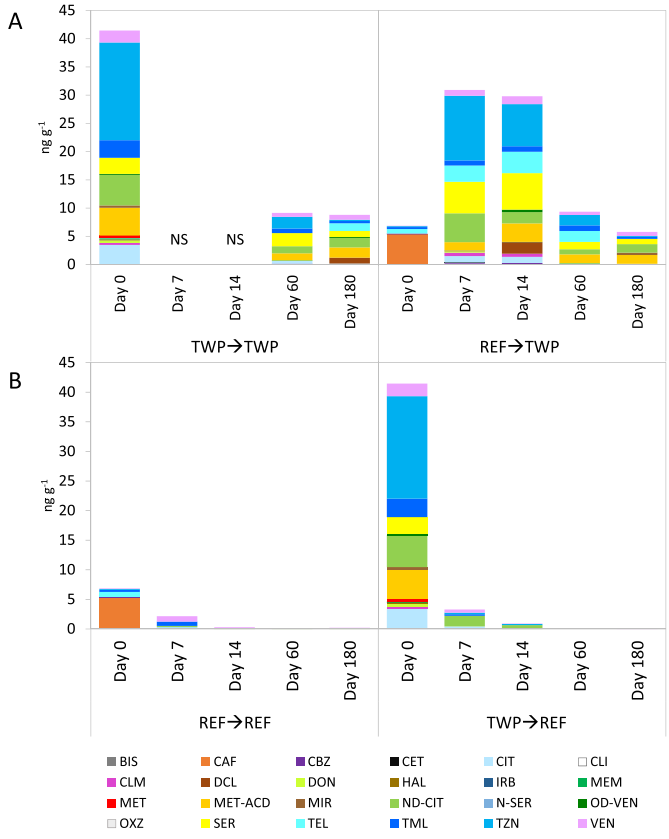


Fig. 2. Concentration of pharmaceuticals and TPs (ng g^{-1} wet weight) identified in the liver of carp with a frequency higher than 50% at each sampling time and treatment. TWP➔TWP – positive control – fish exposed to treated wastewater pond and REF➔TWP – fish transferred from reference pond to treated wastewater pond (A), REF➔REF – negative control – fish adapted to reference pond and TWP➔REF – fish transferred from treated wastewater pond to reference pond (B). BIS – bisoprolol, CAF – caffeine, CBZ – carbamazepine, CET – cetirizine, CIT – citalopram, CLI – clindamycin, CLM – clomipramine, DCL – diclofenac, DON – donepezil, HAL – haloperidol, IRB – irbesartan, MEM – memantine, MET – metoprolol, MET-ACD – metoprolol acid, MIR – mirtazapine, ND-CIT – N-desmethylcitalopram, N-SER – norsertraline, OD-VEN – O-desmethylvenlafaxine, OXZ – oxazepam, SER – sertraline, TEL – telmisartan, TML – tramadol, TZN – trazodone, VEN – venlafaxine, NS – not sampled.

categorised into three therapeutic groups: antipsychotics (mainly antidepressants), cardiovascular drugs (beta-blockers), and nonsteroidal anti-inflammatory drugs (NSAIDs) with a predominance of trazodone, N-desmethylcitalopram, sertraline, metoprolol acid, telmisartan, diclofenac, citalopram, venlafaxine, and tramadol (SM4).

Nineteen PhACs were found in the fish liver from the REF→REF and TWP→REF treatments (Fig. 2B, SM5) and reached a sum of average concentrations of approximately 1–3 ng g^{-1} on Days 7 and 14 (within the short phase of the exposure). Caffeine, telmisartan, tramadol, citalopram, and venlafaxine dominated in the REF→REF treatment, and N-desmethylcitalopram, citalopram, and tramadol prevailed in the TWP→REF treatment. The long phase of the exposure showed only low background concentrations of several pharmaceuticals below 0.5 ng g^{-1}, except for caffeine and metoprolol acid with a 10% frequency in samples.

Principal component analysis (PCA) of fish samples and pharmaceutical response variables displayed two main ordination axes explaining 73.2% of the total variance. In the ordination plot (Fig. 3A), the samples were distributed in four separate clusters almost by PC1, accounting for 65.7% of the total variance. PCA separated treatment TWP→TWP (Day 0) from all REF→REF and TWP→REF groups. Similarly, REF→TWP (Days 7 and 14) fish under short exposure in the TWP were separated near the TWP→TWP (Day 0) group. The groups under long exposure in TWP were distributed between short-exposure REF→TWPand all groups of REF→REF treatment. The main PhACs that separated samples by PC1 (Fig. 3B) accounting for the highest fraction of variation in individual response variables explained by the first axis (given in SM6) were trazodone, sertraline, N-desmethylcitalopram, citalopram, venlafaxine (antidepressants and one metabolite of citalopram), tramadol (an antipsychotic drug), metoprolol acid (a metabolite of beta-blockers metoprolol and atenolol), and clomipramine, haloperidol, and memantine (antipsychotics).

3.4. Metabolome and exogenous compounds in fish liver

In total, 9820 compounds, including endogenous metabolites and exogenous chemicals from the environment, were detected in the fish liver at all treatments and sampling times. The differential analysis, which included all sampling times, revealed elevated/lowered compounds compared to the negative control REF→REF: 1947/545 in TWP→TWP, 365/120 in REF→TWP, and 647/428 in TWP→REF treatment. Compared to positive control TWP→TWP, the analysis found 323/595 elevated/lowered compounds in REF→TWP treatment. All PCA based on comparisons of transformed data of elevated and lowered compounds between two treatments at all sampling times produced four ordination plots with the first two axes explaining the highest percentage of the total variance (Fig. 4). PCA comparing differences between the TWP→TWP and REF→REF metabolomes (Fig. 4A) extracted the first two axes explaining 52.1% of the total variance. Both treatments (negative and positive controls) were diagonal from the left lower corner to the right upper corner. The REF→REF cases were separated from each other according to sampling days: REF→REF on Day 60 had a different metabolome compared to Day 0, and on the contrary, Day 180 was closer to the start of the experiment. On the other hand, TWP→TWP samples were more scattered diagonally without a clear separation of the 60-day exposure cohort. All exposure groups from the same treatment (negative and positive controls) were separated diagonally to the left upper corner and to the right lower corner, respectively.

The metabolic profiles of fish restocked from the reference pond to TWP differed from both exposed and control fish in the short exposure phase. By a long exposure phase of 60 days, one-half of the samples were grouped, but the others were spread and dissimilar to each other. By 180 days, the metabolic profiles were more similar to those of already adapted fish from the TWP and reference ponds. The PCA ordination plot with REF→TWP and TWP→TWP treatment sample score comparison is displayed in Fig. 4B. Because of missing fish samples for analyses

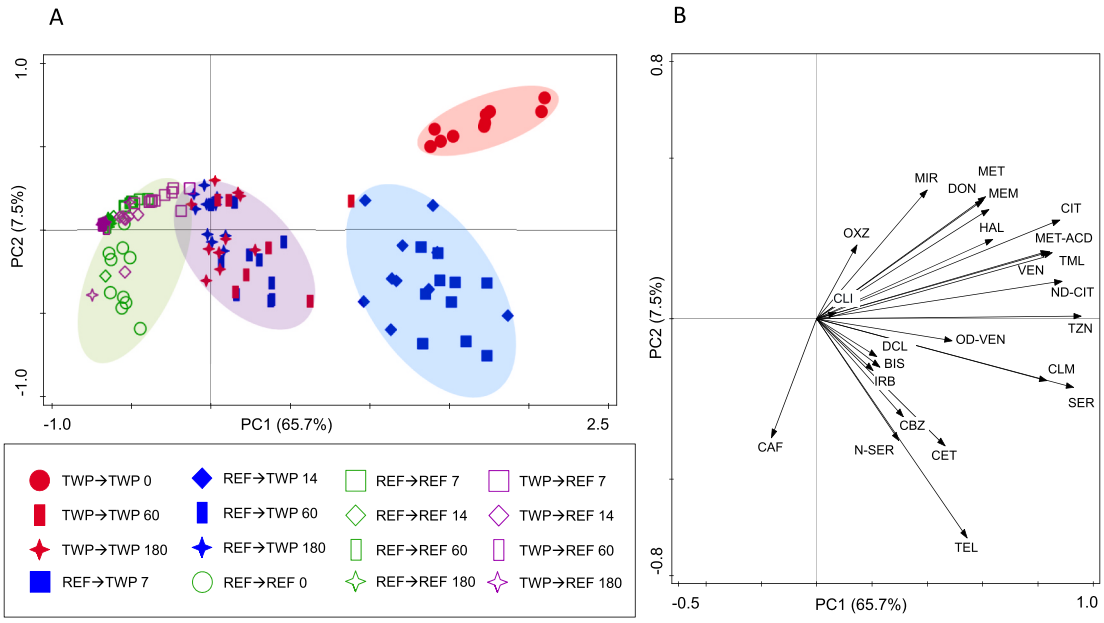


Fig. 3. Principal component analysis (PCA) ordination plots – projection of cases (A) and projection of variables (B). Sample groups belonged to four treatments with a given sampling day. Samples with low intersample distances are encircled for better inspection of individual clusters. Response variables are log-transformed values of pharmaceutical and TP concentrations positively identified in fish liver. The length of the arrow reflects the quality of fit. Abbreviations are the same as for Fig. 2.

in TWP→TWP groups on Days 7 and 14, we also compared REF→TWP vs. REF→REF treatment sample scores (Fig. 4C).

The PCA, comparing TWP→REF and REF→REF metabolic profiles (Fig. 4D), showed two PCs explaining 47.7% of the total variance. Similar to the previous PCA (Fig. 4C), the REF→REF cases were grouped into one cluster at the beginning and within the short phase of the experiment (Days 0, 7, and 14). The REF→REF Day 60 group was separated from the other treatments but close to the dispersed TWP→REF Day 60 group. Moreover, the REF→REF Day 180 group was not strictly distinguished from the three TWP→REF groups on Days 7, 14, and 180.

The PC1 regression coefficients (based on PCA of all samples, given in SM7) did not correlate with morphometric indices. The results of the partial correlation test between PC1 regression coefficients and FCF and HSI showed only four cases with correlation coefficients accounting for at least 0.6 if positively or less than −0.6 if negatively correlated with morphological indices (SM8). We did not perform any correlation test of regression coefficients of PC2 due to the low percentage of explained variance with the second factor (8.1%).

3.5. Antioxidant enzymes and lipid peroxidation

The activities of antioxidant enzymes and LPO in fish liver, kidney, intestine, and gill from the four treatments were characterised by varying values at five sampling times. Complete results are listed in the supplementary material (SM9). The standardisation of the biomarker activities and evaluation of biomarker deviation index (A_i) revealed induction/inhibition effects of oxidative stress in individual tissues of exposed treatments compared to the control (Fig. 5). These evaluation steps determined the basal line centred on zero presenting the negative control treatment. Fish hepatic and renal A_i in the positive control group did not show equal lines with the negative control group in any biomarkers. Fish intestinal indices in the TWP→TWP group were stabilised and nearly repeated the REF→REF group values except for GST and CAT on Day 0 (inhibition) and CAT on Day 60 (induction). Although TWP→TWP fish gills showed similar A_i to the REF→REF group, particularly on Day 60 (except for slight inhibition in GR), gill biomarkers of glutathione-related enzymes were induced in the TWP→TWP group on Day 180. Biomarker deviation indices calculated under REF→TWP and TWP→REF treatments differed in all tissues and sampling times from the REF→REF group. Comparable trends in A_i values were observed in the REF→TWP and TWP→REF groups in the liver, kidney, and gill (all sampling days), except for the opposite effects in GPx indices in the liver on Days 7, 14, and 60. Fish intestinal and gill biomarkers on Day 7 showed the same trends of induction or inhibition in both the REF→TWP and TWP→REF groups, but indices in the REF→TWP group reached more profound extremes than those in the TWP→REF group.

The integrated biomarker response summarising the induction and inhibition of antioxidative enzymes and TBARS showed differences among the TWP→TWP, REF→TWP, and TWP→REF groups with respect to the REF→REF group (Fig. 6). While IBRv2 had a decreasing trend in TWP→TWP samples (from 30.2 to 20.1), the highest IBRv2 was calculated on Days 7 and 60 in REF→TWP samples (both 32.4). Conversely, the highest IBRv2 was calculated on Day 14 in TWP→REF samples (37.9), although Days 7 and 60 showed low values, totalling 24.6 and 27.6, respectively. In all treatments, IBRv2 on Day 180 indicated comparably low levels from 23.2 to 24.1.

4. Discussion

Treated wastewater ponds have been found to be mediators of natural tertiary treatment of WWTP effluent to reduce the dispersion of contaminants of emerging concern before releasing into watercourses (Fedorova et al., 2022; Grabicová et al., 2020). Such recycled water used in aquaculture for its nutrient-rich status could bring potential human health risks (Jana et al., 2018). The present study results revealed the changes in PhAC concentrations and total metabolomic compounds in the fish liver and oxidative stress indicators in four fish tissues in response to alterations in water pollution levels. Knowledge about metabolic changes in fish as a consequence of exposure to CECs can support aquaculture practices if sufficient holding times are identified to depurate contaminants from organisms before shipment from producer to consumer (Brooks and Conkle, 2019).

4.1. Pharmaceutically active compounds in fish liver

The concentrations of detected PhACs in the fish liver in each treatment showed that the target water pollution level for fish breeding

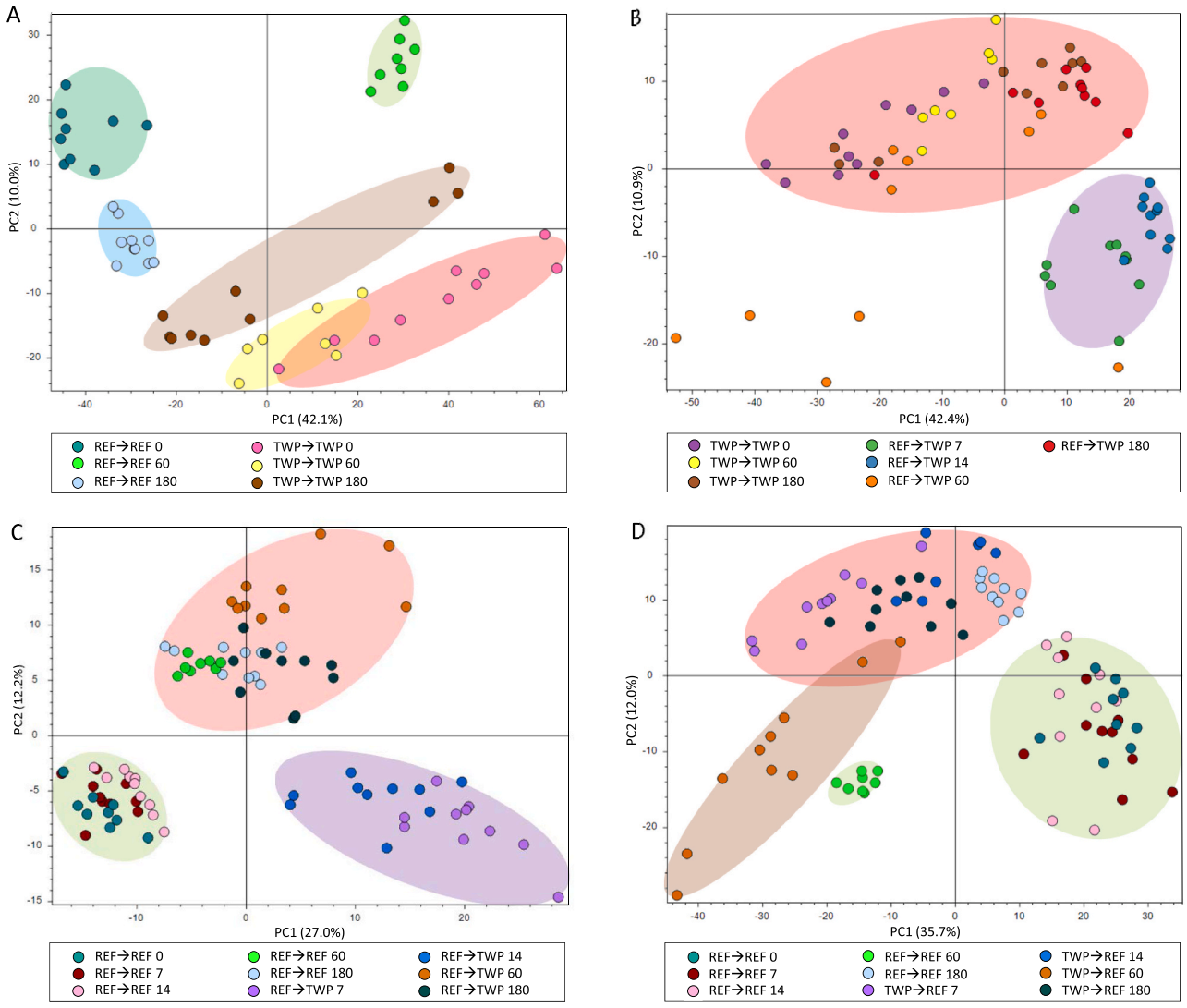


Fig. 4. Ordination plots of sample distribution after principal component analysis of metabolic compounds in liver samples from fish belonging to four different treatments with given sampling days. Different comparisons were performed after differential analysis of elevated and lowered compounds determined in experimental treatments: REF➔REF and TWP➔TWP (A) TWP➔TWP and REF➔TWP (B) REF➔REF and REF➔TWP (C) REF➔REF and TWP➔REF (D). Samples with low intersample distances are encircled for better inspection of individual clusters. Abbreviations are the same as for Fig. 2.

was more important than the original level. Pharmaceutically active compounds were taken up in fish liver in the REF→TWP group relocated to TWP (up to 30 ng g^{-1}) relatively quickly within seven days. During this period, the uptake of pharmaceuticals by fish achieved a steady-state concentration due to the active elimination of metabolites. This finding corresponds to previous bioaccumulation studies carried out within 7–14 days (Garcia-Galan et al., 2017; Scott et al., 2019). High concentrations of antidepressants detected in positive control (Day 0) and REF→TWP (Days 7 and 14) contributed to significant differentiation of these two groups among treatments. As a bottom feeder, common carp can likely uptake antipsychotics from plankton and benthic organisms by swirling in sediment (Grabicová et al., 2020). The present study showed that antipsychotics were the most abundant therapeutic group in TWP sediment (SM3). The concentrations of PhACs decreased by increasing fish body weight from all treatments, despite stable PhAC levels determined in water during the whole experiment. This fact could also explain the low differentiation of sample groups between the TWP and reference pond at the end of the experiment.

Bioaccumulation and elimination of PhACs in fish generally depend on the lipophilicity of the exogenous compounds taken up into organism (Armitage et al., 2017). Many pharmaceuticals common in aquatic systems receiving discharges of municipal water are sparingly water-soluble compounds. Pharmaceuticals are metabolised through the enzymatic system, including cytochrome P450, which catalyses oxidation, hydrolysis, and reduction of lipophilic molecules (Sono et al., 1996). The next conjugation step increases the compound hydrophilicity and facilitates membrane transfer and drug elimination (González-González et al., 2021). Furthermore, conjugated compounds can form reactive xenobiotic electrophiles that bind covalently to macromolecules as nucleotide acid and protein adducts (David Josephy et al., 2005). In the present study, 99.5% of liver-detected PhACs were metabolised or eliminated after fish relocation for up to 14 days except for caffeine and other background compounds detected in the negative control group (REF→REF). The citalopram transformation product N-desmethylcitalopram, with the highest concentration in the TWP→REF group on Day 14, can represent xenobiotic metabolites arising from liver metabolism. Our previous *in vitro* study showed that citalopram can be metabolised by the S9 fraction of the fish hepatic enzymatic system of cytochrome P450 (CYP1A isoform) and is able to form N-desmethylcitalopram (Burkina et al., 2020). Nevertheless, the origin of N-desmethylcitalopram in organisms might be attributed to the persistence of metabolites, particularly those taken up from severely

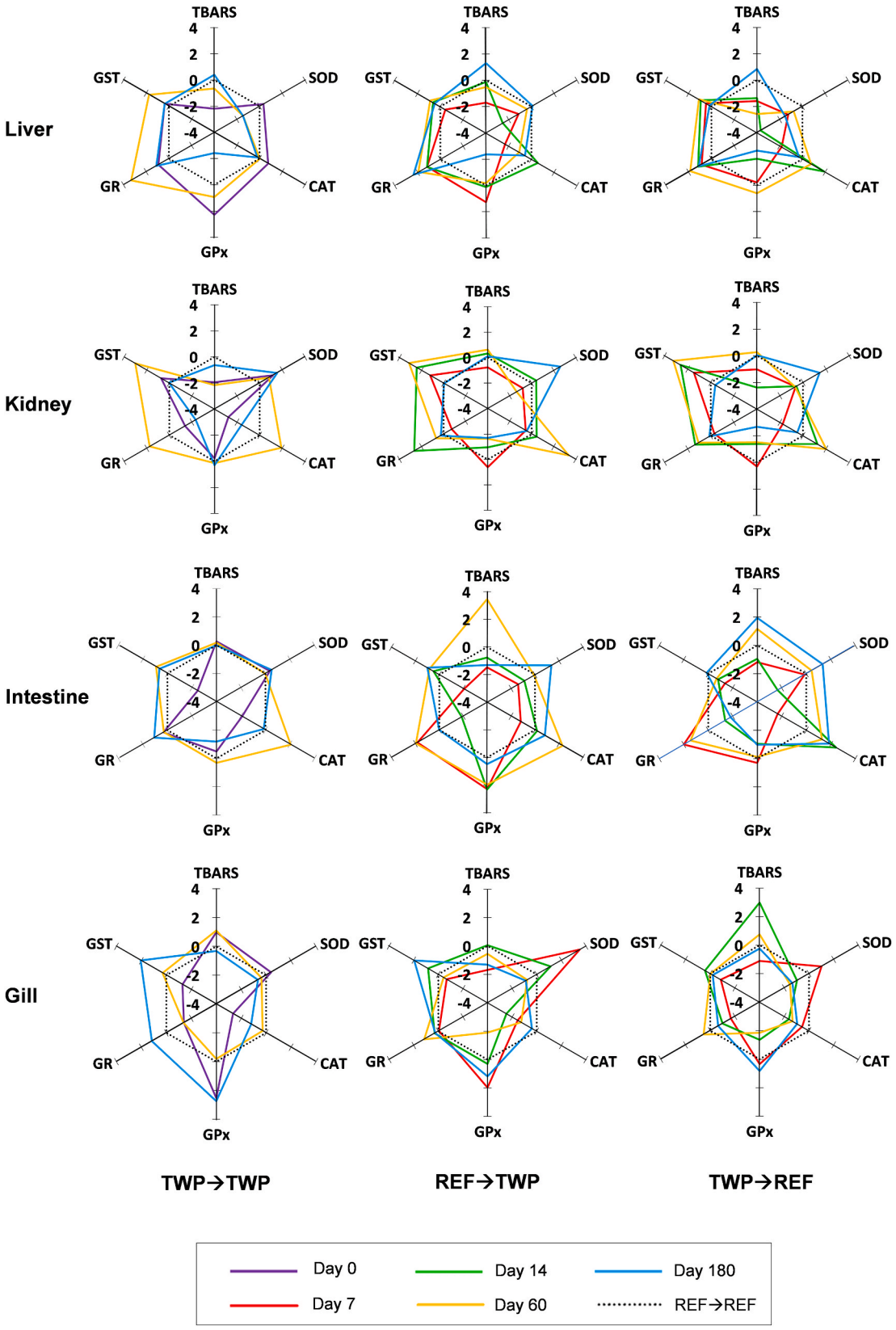


Fig. 5. Star plots of biomarker deviation indices calculated for individual carp tissues (displayed in rows). A positive value of the biomarker deviation index indicates stimulation; a negative value shows inhibition of each biomarker compared to REF→REF treatment, displayed as a zero value. Chart colours represent individual sampling times. Treatment are displayed in columns. Abbreviations are the same as for Fig. 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

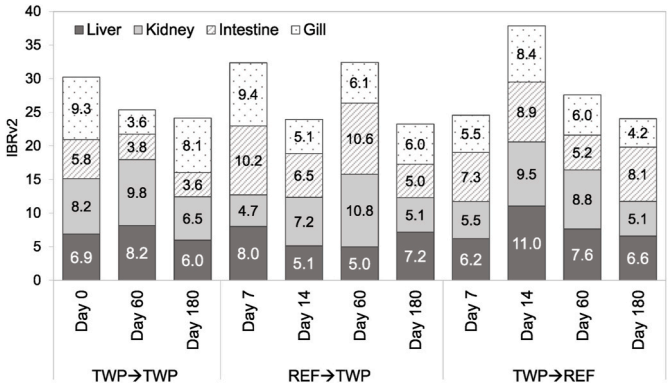


Fig. 6. Integrated biomarker response version 2 (IBRv2) derived from reference values based on zero. IBRv2 explains the sum of absolute values of positive and negative biomarker deviation indices. Fish tissue groups belong to four different treatments with given sampling days. Abbreviations are the same as for Fig. 2.

polluted water.

4.2. Metabolic profile in fish liver

In the fish liver, we determined the metabolic response of carp to exposure to different pollutants. The liver is the primary organ where the detoxification of xenobiotics and their metabolites occurs. The results indicate that almost two thousand elevated compounds in the metabolic profiles of fish originated in the TWP compared to the reference treatment. Meador et al. (2020) reported that exposure to polluted water in the early ontogenetic stages of fish significantly altered metabolic homeostasis compared to unimpacted fish and could decrease energy metabolism and population vitality. Similarly, Jordan et al. (2012) detected altered lipid metabolism in fish liver and increased energy costs due to contaminant exposure. The carp exposed in the present study had an excessive supply of nutrients that balanced the extreme contaminant load within 60 days; however, the final observed FCF was lower in the exposed group than in the unaffected control group.

The metabolic profiles of unaffected fish in the REF→TWP treatment relocated to polluted TWP started to differentiate in a short adaptation phase and lost their native profile. From the number of lowered compounds compared to the reference number, we can deduce that approximately one-half of fish adapted to the exposure around Day 60. In contrast, the other half of the fish responded individually to the pollution load or kept the original metabolome. We cannot exclude that the season, food specificity, and fish body increment influenced the clustering of sample scores in one group at the end of the experiment.

Metabolic profiles of fish restocked from TWP into the reference pond showed that the composition of liver metabolites was more dependent on the sampling season (with specific food intake) than the type of treatment. Seasonally dependent nutrient supply might be a primary factor that indicates similarities between TWP→REF and REF→REF metabolic profiles on Days 60 and 180. Differential analysis showed a three times lower number of elevated compounds in the TWP→REF group than in the positive control group adapted to TWP exposure. This reflects that elevated metabolites were eliminated not earlier than 60 days after the fish restocked to the reference pond. However, the actual return of the metabolome to the reference level cannot be expected. Lower nutritional overlap could lead to perturbations in energy and lipid metabolism due to a shifted metabolic profile, as Jordan et al. (2012) observed. The higher energy investments for depurations were not balanced in TWP→REF treatment by the food supply, as discussed above in the case of positive control treatment.

4.3. Antioxidant enzymes and lipid peroxidation

Oxidative stress biomarkers are suitable indicators of dysfunction or partial damage to defense mechanisms, again ROS (Lushchak, 2011). After enhanced ROS levels, organisms can be subjected to acute or chronic oxidative stress or reach a new quasi-stationary state (Lushchak, 2014). Our results indicated divergent biomarker responses during the short (up to 14 days) and long phases (up to 180 days) of exposure directly dependent on water pollution levels for original and restocked fish.

The short phase of exposure in the REF→TWP treatment, in which fish were restocked from the reference pond to severely polluted TWP, was accompanied by a steep increase in IBRv2 after 7 days of fish transfer. Restocked fish to polluted but nutrient-rich water immediately responded with enzymatic activity changes in the intestinal epithelium. The digestive tract is the first indicator of changes in food supply (De Smet and Blust, 2001) and often water pollution (Dragun et al., 2017). In our study, the increase in FCF compared to FCF in the negative control group indicated a positive response to environmental conditions. The integrated biomarker response decreased on Day 14 and increased again on Day 60 to a status comparable to that on Day 7. We observed a significant increase in LPO in the fish intestine on Day 60, which indicates potential cellular membrane damage. This effect was complemented with high induction of SOD, CAT, and GST activity in the kidney. High activity in the superoxide dismutase enzyme suggests an increase in the dismutation of superoxide anion to molecular oxygen and hydrogen peroxide (Lushchak, 2011). Catalase is responsible for reducing hydrogen peroxide produced from the metabolism of long-chain fatty acids in peroxisomes (Winston and Di Giulio, 1991). Low SOD and CAT activity leads to an accumulation of superoxide and hydrogen peroxide and in turn generates hydroxyl radicals involved in the initiation and propagation of lipid peroxidation (Wang et al., 2016). Complementarily, GST is a multicomponent enzyme family involved in detoxifying xenobiotics and plays an essential role in protecting tissues from lipid peroxidation (Fournier et al., 1992). These findings indicate that fish in our experiment were subjected to the highest imbalance of antioxidative enzymes, probably leading to ROS production in the short exposure phase for up to seven days. The high biomarker response in the long phase of adaptation on Day 60 resulted from lipid peroxidation. High HSI on Days 7 and 60 could be expected since liver metabolise xenobiotics are part of a detoxifying function or indicate an oxidative stress response resulting in altered liver activity, as documented with high GPx on Day 7. However, an integrated view of biomarker responses should be taken into account (Beliaeff and Burgeot, 2002).

The short phase of fish exposure to the TWP→REF treatment, in which carp were restocked from TWP into the reference pond, was accompanied by a high increase in IBRv2 first on Day 14. Fish were confronted by increased oxidative stress associated with a limited food sources than fish in the TWP. This status was followed by the highest intestinal and hepatic IBRv2 and anticipated by low nitrogen and phosphorus in the water. Although the monitoring of zoobenthos was not aimed in this study, the newly acquired food base did not increase the nutrient status of restocked fish. An extensive IBRv2 increase, particularly in the liver, was observed in the long phase of exposure on Day 60, resulting from the utilisation of accumulated energy glycogen stores and leading to the low body increment. Previous studies have shown that diet can affect the activities of antioxidant enzymes in the intestines of common carp, which may be related to the nutritional composition of the diet and the feeding habits of fish (Feng et al., 2013; Wang et al., 2016).

At the end of the experiment, in both the TWP→REF and REF→TWP fish groups, antioxidant enzyme activities achieved similar levels as the control and exposure groups except for GPx activity. This GPx inhibition negatively correlated with increased LPO, particularly in the TWP→REF intestine and REF→TWP kidney groups. Despite the different IBRv2 dynamics of both treatments, we can conclude that fish subjected to

oxidative stress due to transfer to different levels of pollution can reach a new quasi-stationary state between 60 and 180 days to improve or stabilise their physiological status.

5. Conclusions

The present study elucidated the fluctuations in liver metabolic profiles and antioxidant defense systems that accompany acclimation of common carp restocked from the polluted to the reference pond with background pollution and adaptation from the reference to the polluted pond. After acclimation, fish metabolism eliminated pharmaceuticals and their metabolites during the short phase within 14 days, but elevated metabolic profiles persisted for at least 60 days after restocking. Fish transferred from reference to polluted pond increased the antioxidant response and absorbed pharmaceutically active compounds into metabolism within seven days. The final fish weight did not differ among treatments with various culturing practices. Only fish cultured for the whole period in the reference pond indicated significantly higher morphological indices. The restocking practices are commonly applied in carp and other cyprinid aquaculture in Central Europe. Therefore, two weeks are suggested as sufficient holding time for depuration from PhACs residues before shipment of marketed fish from producer to consumer. However, altered metabolic profiles and oxidative stress accompany the restocked fish for more than two months.

Author contribution

Anna Koubová: Data curation, Formal analysis, Investigation, Visualisation, Software, Writing – original draft, Writing – review & editing. Tuyen Van Nguyen: Formal analysis, Writing – review & editing. Kateřina Grabicová: Formal analysis, Writing – review & editing, Viktoriia Burkina: Data curation, Writing – review & editing. Farah Gonul Aydin: Data curation, Formal analysis. Roman Grabic: Conceptualisation, Data curation, Writing – review & editing. Petra Nováková: Formal analysis. Helena Švecová; Formal analysis. Pavel Lepič: Sampling, Methodology. Ganna Fedorova: Writing – review & editing, Tomáš Randák: Conceptualisation, Funding acquisition, Writing – review & editing. Vladimír Žlábek: Funding acquisition, Investigation, Methodology, Project administration, Conceptualisation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2022.119117.

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