Limited oxidative stress in common carp (**Cyprinus carpio, L.**, 1758) exposed to a sublethal tertiary (Cu, Cd and Zn) metal mixture

Limited oxidative stress in common carp (*Cyprinus carpio*, L., 1758) exposed to a sublethal tertiary (Cu, Cd and Zn) metal mixture

Pillet, M.* ; Castaldo, G. ; De Weggheleire, S. ; Bervoets, L. ; Blust, R. and De Boeck, G.

Systemic Physiological and Ecotoxicological Research, Department of Biology, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerp, Belgium

*Corresponding author at: Systemic Physiological and Ecotoxicological Research, Department of Biology, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerp, Belgium. Tel. +32 3 265 3347, Fax +32 3 265 3497

E-mail address: Marion.Pillet@uantwerpen.be

Abstract

Analyzing effects of metal mixtures is important to obtain a realistic understanding of the impact of mixed stress in natural ecosystems. The impact of a one-week exposure to a sublethal metal mixture containing copper (4.8 µg/L), cadmium (2.9 µg/L) and zinc (206.8 µg/L) was evaluated in the common carp (*Cyprinus carpio*). To explore whether this exposure induced oxidative stress or whether defense mechanisms were sufficiently fitting to prevent oxidative stress, indicators of apoptosis (expression of caspase 9 [*CASP*] gene) and of oxidative stress (malondialdehyde [MDA] level and xanthine oxidase [XO] activity) were measured in liver and gills, as well as activities and gene expression of enzymes involved in antioxidant defense (superoxide dismutase [SOD], catalase [CAT], glutathione peroxidase [GPx], glutathione reductase [GR] and glutathione-S-transferase [GST]). The total antioxidative capacity (T-AOC) was also quantified. No proof of oxidative stress was found in either tissue but there was indication of apoptosis in the liver. CAT, GPx, GR and GST total activities were reduced after 7 days, suggesting a potential decrease of glutathione levels and risk of increased free radicals if the exposure would have lasted longer. There were no major changes in the total activities of antioxidant enzymes in the gills, but the relative expression of the genes coding for CAT and GR were triggered, suggesting a response at the transcription level. These results indicate that *C. carpio* is well equipped to handle these levels of metal pollution, at least during short term exposure.

Keywords: antioxidant defenses, *Cyprinus carpio*, metal pollution, mixture stress, oxidative stress

I. Introduction

Metal pollution has increased for decades around the world. During many years, metals from mines and industries were released directly into the aquatic environment (Stohs and Bagchi, 1995), but intensive agriculture, household waste and traffic have been important contributors to metal pollution as well (Burger, 2008; Sevcikova et al., 2011). The metal contamination of the aquatic environment is problematic because these elements are bioaccumulative, non-biodegradable and toxic (Feng et al., 2015). Some metals, such as copper (Cu) or zinc (Zn) are essential elements. They have numerous functions in cellular biochemistry, acting for example as cofactor of superoxide dismutase, cytochrome oxidase and other enzymes (Radi and Matkovics, 1988) and Zn can even act as an antioxidant (Powell, 2000). But ultimately they may become inhibitory or toxic at higher concentrations (Eyckmans et al., 2011; Saddick et al., 2017; Sanchez et al., 2005). Deleterious effects of essential and non-essential metals such as cadmium (Cd) have been investigated on diverse aquatic organisms in numerous studies; e.g. from algae (Jamers et al., 2013) or invertebrates (Benali et al., 2017; Gaete et al., 2017; Jerome et al., 2017; Kerambrun et al., 2016), to fish species (Giguère et al., 2005; Hansen et al., 2006a; Hansen et al., 2007; Hansen et al., 2006b; Komjarova and Bury, 2014; Li et al., 2013). Each metal has different effects on organisms and these impacts vary according to the species and the metal bioavailable concentration. The common point of these three metals is their toxicity associated with the induction of oxidative stress. For example, excessive Cu can alter protein mechanisms promoting oxidative stress (Grosell and Wood, 2002) or interfere with metabolic pathways such as the Krebs cycle (Couture and Rajender Kumar, 2003; dos Santos Carvalho and Fernandes, 2008; Handy, 2003). Excessive zinc concentration is known to induce oxidative stress (Zheng et al., 2016) and to lead to mortality (Bengtsson, 1974). Cd disturbs the antioxidant (Stohs and Bagchi, 1995)

system, interferes with gene regulation (Wang et al., 2004), induces apoptosis (Gonzalez et al., 2006), inhibits the electron transport chain and thus increases ROS production and reduces ATP production (Livingstone, 2001; Wood et al., 2011b).

Highly reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion radical ($O_2^{-\bullet}$) or hydroxyl radical ($OH^\bullet$), are metabolically produced under normal conditions as side products of aerobic metabolism, but also in cytosol by some oxidases such as xanthine oxidase (XO) (Halliwell and Gutteridge, 1999; Livingstone, 2001). Oxidative stress appears when the rate of ROS generation exceeds the antioxidant defense system (Livingstone, 2001; Lushchak, 2011). The disturbance of the steady-state ROS concentration is due to several parameters: inhibition of antioxidant enzymes, alteration of the mitochondrial electron transfer chain, additional formation of ROS (via the Fenton reaction) or depletion of cellular glutathione (GSH) (Beg et al., 2015; Huang et al., 2007; Wang et al., 2004). Oxidative stress might cause osmoregulatory dysfunctions, tissues damage via the oxidation of protein and lipids (lipid peroxidation) and alteration of gene expression (Livingstone, 2001; Lushchak, 2011; Radi and Matkovics, 1988). When cells undergo severe oxidative stress, they often undergo apoptosis (Pellegrini and Baldari, 2009). Apoptosis is induced by distinct intracellular signalling pathways molecules and transmembrane receptors that lead to its execution (Chu et al., 2018; Song et al., 2017). Some major molecules, such as caspase 9 (CASP), mediated apoptosis through the mitochondrial pathway (Wang et al. 2018; Wang et al. 2019). Antioxidant defense systems, including various antioxidant enzymes, are present to prevent ROS from causing adverse effects. Superoxide dismutase (SOD, EC 1.15.1.1) is the first line of defense by scavenging $O_2^{-\bullet}$. Catalase (CAT, EC 1.11.1.6) and glutathione peroxidase (GPx, EC 1.11.1.9) convert H_2O_2 originated from the previous reaction into water (Atli and Canli, 2010; Weydert and Cullen, 2010). These three enzymes deal directly with ROS, but other enzymes contribute to the maintenance of the level of

GSH, another antioxidant. Glutathione reductase (GR, EC 1.6.4.2) catalyses the reduction of glutathione disulphide (GSSG) to maintain GSH/GSSG ratio, and glutathione-S-transferase (GST, EC 2.5.1.18) metabolizes lipid hydroperoxides (Dautremepuits et al., 2009; Weydert and Cullen, 2010). These antioxidant enzymes are considered sensitive bioindicators of metal contamination but they can vary according to the metal type and concentration (Atli and Canli, 2010; Huang et al., 2007).

Our first objective was to investigate the effect of mixed metal stress (Cu/Zn/Cd) on oxidative stress and antioxidant defense processes in common carp (*Cyprinus carpio*). We hypothesized that an increase of these two parameters due to metal exposure would occur. In a natural environment, organisms are exposed to diverse contaminants with different toxic potentials. Therefore it is important to assess the interaction of mixtures with living species (Altenburger et al., 2004; Beg et al., 2015). Secondly, the present study aimed to understand the time course of these processes over a week of exposure in the two main target organs, liver and gills of *C. carpio*, hypothesizing that the damage would accumulate and therefore responses would become more important with time. *Cyprinus carpio* is a freshwater fish species which is economically important around the world, and often used as bioindicator in environmental pollution studies (Altun et al., 2017; Rajeshkumar et al., 2017). Its populations can be threatened by severe metal contamination (Ahmad et al., 2015). This species has been well studied to assess among others the effects of metal pollution on bioaccumulation (Bervoets et al., 2009), apoptosis (Cols Vidal et al., 2008; Gao et al., 2013b), energy status (De Boeck et al., 1995b; Kunwar et al., 2009), lipid metabolism (Meng et al., 2018), immune function (Zhang et al., 2017) or oxidative stress (Cortes-Diaz et al., 2017; Dugmonits et al., 2013; García-Medina et al., 2017). Our study is the first to examine the combined effects of Cu, Zn and Cd mixtures on *C. carpio*. Here, we measured indicators of apoptosis (expression of *CASP*, an apoptosis pathway signaling molecule)

105 and of oxidative stress (MDA content analyses and XO activity), as well as the total antioxidative
106 capacity (T-AOC) and several enzymatic biomarkers (SOD, CAT, GPx, GR and GST).
107

II. Material and methods

A. Experimental animals

Fish individuals (around 4 months old) were obtained from the fish hatchery at the Agricultural University of Wageningen (The Netherlands). They were kept in 1000 L aquaria at 20°C and under 12 h light and 12 h dark photoperiod conditions for eight months before being used in experiments. For acclimation, 200 individuals were distributed into four 200 L polyethylene tanks (50 fish per tank) supplied with medium hard water (pH 8.2 ± 0.2). As defined by the US Environmental Protection Agency (USEPA, 2002), medium hard water was reconstituted with deionized tap water (Aqualab, VWR International, Leuven, Belgium) supplemented with 96 mg/L $NaHCO_3$, 60 mg/L $CaSO_4.2H_2O$, 123 mg/L $MgSO_4.7H_2O$ and 4 mg/L KCl (VWR Chemicals). Each tank was equipped with a recirculating water system and water quality was ensured through a biofilter containing wadding, glass stones and plastic balls. In each tank, oxygen was provided by an individual air stone, the temperature was maintained at 20°C and the photoperiod was 12 h light and 12 h dark. Fish were maintained in these tanks for 2 weeks before the experiment and were fed *ad libitum* once a day with commercial pellets (*Hikari*® *Staple*™, Klundert, The Netherlands) during this period. In addition, fish were starved two days prior the experiment.

Experimental methods complied with regulation of the Federation of European Laboratory Animal Science Associations and were approved by the local ethics committee, University of Antwerp (Permit Number: 2015-94, Project 32252).

B. Experimental design

For the metal exposure, the experimental scheme is presented in Fig. 1. Fish (length = 65.2 ± 7.4 mm; weight = 3.6 ± 1.2 g) were exposed to a metal mixture (Cu: 4.8 µg/L; Cd: 2.9 µg/L and Zn: 206.8 µg/L) for 1 day, 3 days and one week. The used concentrations correspond to the 10% of the 96 h LC_{50} of the fish, determined on a similar batch of fish with a size of 2.6 ± 1.0 g in medium hard water at 20°C (Delahaut et al., personal communication). The experimental set up consisted of ten 10 L double-walled polypropylene buckets (5 for control and 5 for treatment, 6 fish per bucket) filled with 9 L of medium hard water. Stock solutions of copper sulfate (0.22 g/L, Sigma), cadmium chloride (0.13 g/L, Merck) and zinc chloride (9.31 g/L, Sigma) were prepared in MilliQ water and added to the exposure water to reach the desired concentrations. In each bucket, oxygen was provided by an individual air stone. To avoid accumulation of waste products (such as ammonia), 8 L of water were replaced with fresh water containing the same concentration of metals each day. To minimize disturbance to the fish, the inner bucket was lifted from the outer bucket. The fish and one liter of water stayed behind in the inner bucket, and the remaining 8 L of water in the outer bucket could easily be replaced after which the inner bucket was reinserted. To check the stability of the conditions, 10 mL of water were sampled in each bucket every day, before and after the water replacement. Concentrations of metals were measured in the water samples by inductively coupled plasma mass spectrometry (7700x ICP-MS, Agilent Technologies, Santa Clara, CA, USA) after acidification of the sample by adding 150 µL of nitric acid (67-69%, trace metal grade, Fisher Chemical). The recorded metals concentrations during the experiment ranged from 0 to 1.5 µg/L for Cu, 0 to 0.6 µg/L for Cd and 0 to 6.3 µg/L for Zn in control buckets and from 2 to 7.2 µg/L for Cu, 0.4 to 4.5 µg/L for Cd and 78.6 to 466 µg/L for Zn in treatment buckets. Fish were not fed during the experiment to avoid

differences in appetite that could have made difficult the comparisons among treatments and increase the inter-individuals variability.

At day 1, 3 and 7, fish were anaesthetized with neutralized MS222 (pH 7.0, ethyl 3-aminobenzoate methane-sulfonic acid, 300 mg/L, Acros Organics, Geel, Belgium). Liver and gill tissues were sampled, immediately frozen in liquid nitrogen and stored at -80°C until further analysis. Ten exposed fish and 10 control fish (two from each bucket) were sampled at each exposure time.

C. Enzyme activities

Liver and gill samples were weighted (wet mass) and homogenized in 10 volumes of phosphate buffered saline solution (PBS, pH 7.5) containing 0.1% Triton X-100 and 1 mM methylene diamine tetra-acetic acid (EDTA). Samples were homogenized using a MagNALyser (Roche, Vilvoorde, Belgium) and then centrifuged (16000 g) for 30 min. The supernatant was recovered and divided into aliquots for enzyme activity determinations.

The activities of CAT, GPx, SOD, GR, GST (antioxidant defense) and XO (index of oxidative stress) were measured in both tissues. CAT activity was determined according to Aebi (1984) by measuring the rate of decomposition of H_2O_2 at 240 nm. GPx activity was assayed according to Paglia and Valentine (1967) as modified by Janssens et al. (2000), by monitoring the consumption of NADPH at 340 nm. SOD activity was quantified according to Marie et al. (2006) adapted from Flohé and Ötting (1985). A standard curve using known units of SOD enzyme under identical conditions against the percentage of cytochrome c reduction at 550 nm was used. GR activity was determined according to Carlberg and Mannervik (1985) by recording the oxidation of NADPH at 340 nm. GST was analyzed according to Habig et al. (1974) by

measuring the production of GS-DNB at 340 nm. XO activity was assayed according to Beckman et al. (1989) by recording the production of uric acid at 295 nm.

All activity measurements were scaled down for semi-high throughput using a micro-plate reader (Synergy Mx, Biotek Instruments Inc., Vermont, USA). For all enzymatic assays, substrate and cofactor concentrations yielding optimal reaction velocities were used with homogenates diluted to obtain linear reaction slopes for 5 min, at 25°C. Total protein concentrations were determined using the Lowry method modified by Peterson (1977). All chemicals were obtained from Sigma-Aldrich®. Total and specific enzymatic activities were measured and expressed as U (µmol/min)/g of wet tissue and U/mg of protein, respectively. All analyses were performed in duplicate using standard methods adapted for a microplate reader.

D. Total antioxidative capacity

The T-AOC was quantified in tissue homogenates using the ferric reducing ability of plasma (FRAP) method (Benzie and Strain, 1996) and expressed as Trolox (±-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) equivalents. The principle of this method is based on the oxidation of ferrous ions to ferric ions by the activity of hydroperoxides. The reaction results in the formation of a blue compound, when the iron is reduced, which absorbs at 593 nm. Standard curve was obtained by diluting Trolox solution. All analyses were performed in duplicate using standard methods adapted for a microplate reader.

E. Products of oxidative stress

Malondialdehyde (MDA) content, a secondary product of lipid peroxidation, was quantified using the thiobarbituric acid reactive substances (TBARS) assay, according to Devasagayam et

198 al., 2003. The principle of this method is the estimation of the aldehydic products by their ability
199 to react with thiobarbituric acid. The reaction produces pinkish red chromogen thiobarbituric
200 acid-malondialdehyde complexe that is recorded by spectrophotometry at 532 nm. The
201 absorbance at 600 nm was also recorded as a correction factor for nonspecific turbidity. All
202 analyses were performed in duplicate using standard methods adapted for a microplate reader.
203

204 ## F. Gene expression

205 Total RNA was extracted from ~20 mg of tissues using TRIzol™ (Invitrogen, Merelbeke,
206 Belgium) following the manufacturer's instructions and Macedo and Ferreira (2014). Ribonucleic
207 acid quantity and integrity were determined respectively using a NanoDrop spectrophotometer
208 (NanoDrop Technologies, Wilmington, DE) and a 1% agarose gel with ethidium bromide
209 (500 µg/mL). The RNA purity was checked by OD_{260}/OD_{280} nm absorption ratio and was always
210 higher than 1.8. A DNase treatment was applied to 1 µg of extracted RNA using DNase I, RNase
211 free kit from Thermo Fisher Scientific (Waltham, MA, USA). This DNase treated RNA was
212 analyzed with qPCR to check the entire removal of genomic DNA. Immediately after the DNase
213 treatment, RNA was transcribed to cDNA using Reverse Transcriptase Core kit (Eurogentec,
214 Seraing, Belgium). Reverse transcription was performed in a total volume of 10 µL containing
215 4 µL of 5× reaction buffer, 1 µL of RiboLock RNase inhibitor, 2 µL of 10 mM dNTP mix, 1 µL
216 of ReverAid H minus and 100 ng of RNA. The thermal steps of reverse transcription (Eppendorf
217 MasterCycler® Gradient, Hamburg, Germany) were 5 min at 25°C, followed by 60 min at 42°C
218 and 5 min at 70°C. The quantity and purity of cDNA were verified using a NanoDrop
219 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Complementary DNA samples
220 were separated into aliquots and kept frozen at -20°C until further analysis.

Real-time PCR was performed using Takyon™ Low Rox SYBR® MasterMix dTTP Blue kit (Eurogentec, Seraing, Belgium).

The relative expression of 6 target genes (*CAT, SOD, GPx, GR, GST* and *CASP*) was measured in tissue samples (10 per each exposed and control group). Gene encoding for elongation factor 1-alpha (EF1α) was used as reference gene. The mRNA sequences for reference and target genes were available in the GenBank database (Table 1). Oligonucleotides primers were designed using NCBI resources Primer blast (except for *EF1α* and *GR*, already designed by Sinha et al. (2012) and Wu et al. (2014) respectively) and synthesized as highly purified salt-free "OliGold" primers by Eurogentec (Eurogentec, Seraing, Belgium). On test samples of both tissue, each amplicon obtained with the different primers was amplified by real-time PCR using Takyon™ Low Rox SYBR® MasterMix dTTP Blue kit (Eurogentec, Seraing, Belgium). Melting curves were performed to check the presence of unique PCR product in each reaction. Integrity and size of PCR products were verified on 2% agarose gel with ethidium bromide (500 µg/mL). Sequences for these primers are presented in Table 1.

Real-time PCR analyses for each gene were performed in duplicate in a total volume of 20 µL containing 10 µL of Takyon™ master mix, 100 nM of each primer (reverse and forward), 3.5 µL of sterile water and 50 ng of cDNA (Eurogentec, Seraing, Belgium). The thermal cycling of qPCR (Stratagene Mx3005P, Agilent Technologies, Waldbronn, Germany) was initiated with a Takyon™ activation at 95°C for 3 min. Forty-five PCR cycles were then performed, each of which consisted of a denaturation step at 95°C for 10 s. and an annealing/extension step at 62°C for 60 s. A final cycle (95°C for 1 min, 62°C for 30 s. and 95°C for 30 s.) was performed to obtain a dissociation curve.

Quantification cycles (Cq) values were automatically calculated on the log curve for each gene with MxPro QPCR software (Agilent Technologies, Waldbronn, Germany). Stability of EF1α

expressions was tested by two-ways ANOVA in liver (for duration $F_{[2;53]}$ = 2.645 and P = 0.080; for condition $F_{[1;53]}$ = 2.676 and P = 0.108; and for interaction $F_{[2;53]}$ = 1.113 and P = 0.336) and gills (for duration $F_{[2;54]}$ = 1.007 and P = 0.372; for condition $F_{[1;54]}$ = 2.464 and P = 0.122; and for interaction $F_{[2;54]}$ = 0.882 and P = 0.420).

Quantification of the target genes relatively to the reference genes was calculated following the Pfaffl method (Pfaffl, 2001; Pfaffl et al., 2002) but using the formula:

$$gene\ expression\ ratio = \frac{\left(E_{target}\right)^{\Delta Cq_{target}}}{\left(E_{ref}\right)^{\Delta Cq_{ref}}}$$

where Cq value corresponds to the number of cycles at which the fluorescence emission monitored in real-time exceeded the threshold limit. ΔCq is the mean Cq of the control group (individuals non exposed to metals) minus the Cq of each sample. E is PCR efficiency (Table 1) determined by standard curve using serial dilution of cDNA and calculated according to the equation:

$$E = 10^{\left(\frac{-1}{slope}\right)}$$

Results for the gene expression are expressed as fold expression relative to *EF1α*.

G. Statistical analysis

Normality and homogeneity of variances were verified by Shapiro and Levene tests. Data were log or square transformed to avoid heteroscedasticity when necessary. Two-way ANOVAs were

267 used to test the effects of metal exposure and duration of this exposure on all measured
268 parameters in *C. carpio*. When significant effects were found, a posteriori Tukey tests were used
269 to compare means ($\alpha = 0.05$). Analyses for simple main effects were applied to the data when
270 there was no interaction effect (Tybout and Sternthal, 2001). Student t-tests were applied to test
271 the effect of exposure at each sampling time. One-way ANOVAs were performed to analyze the
272 effect of exposure duration for each condition, followed by Tukey post-hoc test when a
273 significant effect was found.
274 Pearson's correlations between total and specific enzymatic activity responses were verified for
275 all enzymes.
276 When normalization of the data was not possible, non-parametric equivalent tests were used. All
277 statistical analyses were performed with R software.
278

III. Results

Specific and total activities measured in both tissues of *C. carpio* exposed to the metal mixture are significantly correlated (R between 0.64 and 0.98 depending of the enzyme, $P<0.001$). Total activity is presented here because it is best suited to be related to transcription activity. Specific activities and others parameters expressed per mg of protein can be found in supplementary Tables A and B.

A. Protein content

Compared to the control group, fish exposed to metals contained significantly lower protein concentrations after 7 days, 14% and 24% in liver and gills respectively (Fig. 2). The protein concentration for the control group was stable during the experiment. It was more variable in the group exposed to metals: in the liver, the drop in protein levels was gradual and therefore the concentration in the exposed fish was significantly higher at day 1 compared to the end of the experiment, whereas in the gills the protein concentration were variable with a transient significant increase at day 3 compared to day the exposed fish at day 1 and 7.

B. Indicators of apoptosis and oxidative stress

The relative expression of *CASP* was 2.6 fold significantly higher in *C. carpio* exposed to metals compared to the control group after one day (Fig. 3A). However, due to the high variability within the exposed groups, there was no difference between exposed and control fish for the other sampling dates. In gills, metal exposure did not significantly impact the relative expression of *CASP* (Fig. 3B). However, the duration of the exposure had an effect within the group exposed

301 to metals. In this group, *CASP* was significantly more expressed at day 1 compared to day 7, with
302 intermediate expression at day 3.
303 In the liver and the gills of *C. carpio*, exposure to metal did not affect the total activity of XO or
304 TBARS, when we compared the control group to the exposed group (Fig. 4). However, in both
305 tissues, the duration of the exposure had a significant impact on the TBAR level in control and
306 exposed groups. These levels were slightly decreased after 7 days in both groups.
307 The duration of metal exposure also affected the total activity of XO in the gills of fish in the
308 group exposed to metal. The XO total activity showed a tendency to decrease progressively in
309 both control and exposed group, but in the exposed group this became a significant difference
310 between day 3 and 7.
311

312 ## C. Antioxidant defense

313 In the liver and the gills of *C. carpio*, the T-AOC remained stable during the exposure to the
314 metal mixture (Fig. 5).
315 In the liver of *C. carpio*, SOD total activity (Table 2) and relative expression (Table 3) remained
316 unchanged during the experiment. The total activity of CAT was transiently but significantly
317 reduced by 33% in the group exposed to metal compared to the control group at day 3, but
318 remained stable at day 1 and 7 (Table 2). Even if no difference was observed between the control
319 and the exposed group, the relative expression of *CAT* within the exposed group increased
320 progressively during the experiment and became significantly higher at day 7 (Table 3).
321 The glutathione enzymatic system was impacted by the metal exposure in the liver. All three
322 enzymes involved (GPx, GR and GST) showed significant reduced total activities after several
323 days in fish exposed to metals compared to the control group (Table 2). The total activities of

GPx and GST decreased by respectively 51% and 29% after 7 days, whereas the total activity of GR started to decrease after 3 days (reduction by 31% at day 3 and 37% at day 7). Within the exposed group, the progressive decrease of GR total activity was marked by a significant difference between total activity at day 1 and 7. Significant changes were also observed in the relative expression of *GPx, GR* and *GST* but only within the group exposed to metals (Table 3). *GPx* and *GST* presented a higher relative expression at days 3 and 7 compared to day 1. The same pattern was observed for the relative expression of *GR*, except that the increase was more progressive and that the difference between days of exposure was significant only between day 1 and day 7 (Table 3).

In the gills of *C. carpio*, we observed less response. The exposure to metal had no effect on the total activities of SOD and CAT because no difference was observed between control and exposed groups during the experiment (Table 2). However, the duration of the exposure decreased significantly the SOD total activity within the exposed group after 7 days. The relative expression of *SOD* remained unchanged during the experiment (Table 3). On the contrary, metal exposure increased significantly the relative expression of *CAT* after 3 days (Table 3).

Concerning the glutathione enzymatic system in the gills, metal exposure had no effect on the total activities of GPx, GR and GST. However, duration of the experiment affected the GPx total activity in the exposed group and the GST total activity in the control group (Table 2). In both situations, total activities were significantly suppressed at day 7 compared to day 3, with intermediate total activity at day 1. The relative expressions of *GPx* and *GST* remained unchanged during the experiment (Table 3). At day 3, the group exposed to metal had a significantly higher relative expression of *GR* compared to controls (Table 3).

IV. Discussion

In this study, *C. carpio* were exposed during one week to a mixture of metal pollutants. The concentrations used in the mixture (Cu: 4.8 µg/L; Cd: 2.9 µg/L and Zn: 206.8 µg/L) were equivalent to 10% of their individual 96 h LC_{50} measured for *C. carpio* (Delahaut et al., personal communication). No mortality was observed during the experiment, confirming that concentrations used, taking into account possible additive or synergistic effects, were also sublethal when combined in a mixture. These concentrations are environmentally relevant and correspond to the concentrations recorded in the field. In Flanders, the Flemish Environmental Agency (VMM) measured concentrations, independent of each other, ranging from 1.27 to 34.32 µg/L for Cu, 0.05 to 3.37µg/L for Cd and 7.84 to 330.17 µg/L for Zn (VMM, 2014). In a parallel experiment with the same size-class *C. carpio* exposed to these concentrations of metals, accumulation of Cu, Cd and Zn was measured (Castaldo et al., personal communication). While Zn did not seem to accumulate to any significant level in the tissues, Cu and Cd concentrations increased after the exposure to metals. The same kind of results was observed in fish species along a polymetallic gradient by Andres et al. (2000). In the present study, Cu was significantly higher in the liver and gills of the *C. carpio* after respectively seven and one day and there was significant and progressive increase of Cd in both tissues from day 1 onwards.

Metal mixture had an impact on the protein content of *C. carpio*. Not only the concentration of protein was reduced after a week in the group exposed to metals, but the variability during the experiment was much more important in this group. Previous studies have shown inhibitory effects of metals on protein content. Radi and Matkovics (1988) recorded a decrease of protein content in tissue of *C. carpio* in presence of Cu (from 5 to 50 ppm) and they linked this effect

370 with the appearance of lipid peroxidation. However, no sign of lipid peroxidation was recorded in
371 the present study. The decrease in protein content could also be the result of the breakdown of
372 protein into amino-acids to meet higher energy demands during stressing conditions, as suggested
373 by Tripathi et al. (2012) who also reported a decrease of protein content in *Colisa fasciatus* after
374 30 days of sublethal 6.22 mg/L Zn exposure (corresponding to 10% of the LC_{50} of this species).
375 De Boeck et al. (1995a) also showed an increase in the use of protein as demonstrated by a higher
376 ammonia quotient in *C. carpio* exposed to 0.34 and 0.84 µmol/L Cu. Disturbance of
377 osmoregulation, a well-known impact of Cu (Grosell and Wood, 2002), could also increase the
378 water content of tissues and decrease the concentration of proteins on a per weight basis. All
379 these hypotheses can explain our results. It implies that the mixture of metals used in our
380 experiment, even at relatively low concentrations, actually did become stressful for *C. carpio*
381 after a few days.
382 In our study, the variation of the protein concentration had an effect on the specific enzymatic
383 activity (expressed as U/mg of protein) results, as significant differences observed with in the
384 total activity results were no longer seen in the specific activity result. The higher variability and
385 the decrease of protein at day 7, either due to inhibitory effect of metal, higher energy demand in
386 stressing conditions or a higher water content in the tissue, counteracted the decrease of total
387 activity often observed at the same day.
388
389 Some signs of apoptosis signaling were observed at the beginning of the experiment, especially in
390 liver, but *C. carpio* acclimated and *CASP* expression returned to normal after a few days. Hossain
391 et al. (2009), in cell culture, and Risso-de Faverney et al. (2004), in *C. carpio*, found that Cd (at
392 respectively 25-100 µM and 2-10 µM), especially at low concentrations, induces an activation of
393 caspase 9. Gonzalez et al. (2006) also discovered that apoptotic genes were up-regulated in

394 *D. rerio* exposed to 2-10 µg/L Cd. These results from previous studies confirm that metals can
395 increase *CASP* expression, as seen in the present study. Apoptosis is an important regulatory
396 process for the cell, by destroying damaged or infected cells that may interfere with normal
397 function (Gao et al., 2013a). In previous single metal exposure experiments in fish, apoptosis has
398 been associated with oxidative stress and DNA damage (Choi et al., 2010; Risso-de Faverney et
399 al., 2004; Zheng et al., 2014). In our study, DNA damage was not measured and we did not
400 notice any sign of lipid peroxidation. Other oxiradicals (such as H_2O_2 or $OH^{\bullet}$) could also be
401 responsible for oxidative stress followed by apoptosis. The increase of *CASP*, an initiator of
402 apoptosis, at the beginning of our experiment suggests an increase of apoptosis to reduce
403 accumulation of damaged cells and avoid potential damage; however the cause of apoptosis still
404 needs to be explored in a further study. Apoptosis processes are complex and cannot be explained
405 by analyzing *CASP* expression only. Here, *CASP* is one among other mitochondrial apoptosis
406 pathway signaling molecules (Wang et al., 2018; Wang et al. 2019) and is not an executioner of
407 apoptosis. Nevertheless, measuring *CASP* expression is an interesting first approach to
408 understand apoptosis processes under metal exposure.
409
410 No sign of oxidative stress was observed in the liver and the gills of *C. carpio* during a week of
411 exposure to these concentrations, even if the responses were more variable in the group exposed
412 to metals. The two indices measured, XO activity and lipid peroxidation (MDA) remained
413 unchanged during the experiment. These results are interesting knowing that several previous
414 studies showed an induction of oxidative stress in diverse fish species exposed to higher
415 concentrations of metals. In the liver, *C. carpio* showed elevated $O_2^{-\bullet}$ levels when exposed to 10
416 mg/L Cd^{2+} (Dugmonits et al., 2013), elevated MDA levels when exposed to 0.41-2.06 mg/L Cd
417 or 2.5 µM Cd^{2+} (Jia et al., 2011; Zhang et al., 2017) and increased lipid peroxidation in gills and

liver when exposed to 0.01 mg/L mercury (García-Medina et al., 2017). Lipid peroxidation levels increased with metals in *Channa punctatus* exposed to heavy metals in canals (Javed et al., 2016), in *Galaxias maculatus* exposed to 2.5 and 10 µg/L Cd (McRae et al 2018) and in *Dicentrarchus labrax* kidney when exposed to 500 mM Cd or 200 mM Cu (Roméo et al., 2000). Lipid peroxidation can be highly deleterious, causing organs or tissue damages (Lushchak, 2011). Tissues show different responses facing oxidative stress (Martínez-Álvarez et al., 2005), but gills (directly in contact with environmental stressors) and liver seem more sensitive to it (Dautremepuits et al., 2009). However, the stress applied in our experiment appears to be minor and *C. carpio* is able to avoid oxidative stress during one week at these sublethal concentrations. Although opposite to the basic principle, Eyckmans et al. (2011) found no sign of oxidative stress in adult *C. carpio* exposed to 50 µg/L Cu during a one month exposure and Giguère et al. (2005) found similar results in *Perca flavescens* collected along a metal contamination environmental gradient (0.02-2.3 nmol/L Cu, 0.3-6.7 nmol/L Cd, 10-26000 nmol/L nickel and 20-110 nmol/L Zn) and even associated a reduced lipid peroxidation to an increasing Cu accumulation in the liver. As there was also an accumulation of Cu in a parallel experiment (Castaldo et al., personal communication), the same link could be made about our results. The absence of oxidative stress in our study could be either due to the metal concentrations being too low to increase the production of ROS or to the efficiency of the oxidative defenses, which were clearly responding. These results suggest that, even as a mixture, an exposure to 10% LC_{50} Cu/Cd/Zn (previously measured on the individual metals) could be considered safe for the *C. carpio*, at least during our short term exposure.

To counteract the negative effect of ROS, organisms have antioxidant enzymatic and non-enzymatic defense processes, involving for example SOD, CAT, GPx or reduced glutathione

(GSH) (Halliwell and Gutteridge, 1999; Lushchak, 2011), and all having specific role in the T-AOC. Some of these parameters were measured in our study and the absence of oxidative stress is confirmed by the stability of the T-AOC during the experiment and the response of antioxidant defense system.

The response of antioxidant defenses was measured at the enzymatic and the genomic levels in our study and their response pattern was different at the two levels. At the genomic level in the liver, *CAT, GPx, GR* and *GST* mRNA levels remained stable while total activities decreased, suggesting their regulation may occur at a post-translational level (Hansen et al., 2006b; Zheng et al., 2016). On the other hand, in the gills increases in the expression of *CAT* and *GR* were accompanied by unchanged enzymatic total activities, suggesting they might be regulated at a translational level (Defo et al., 2015). This discrepancy was observed previously with *MT* (Gonzalez et al., 2006), *CAT, SOD* and *GPx* (Banni et al., 2011; Zheng et al., 2016) in *D. rerio* exposed to Cd, or with *SOD* and *CAT* in *S. trutta* exposed waterborne Cu/Zn (Hansen et al., 2006a; Hansen et al., 2007). Several hypotheses can be proposed to explain these differences. It could be explained by a time delay in the responses at different levels, or by an impact of metals on transcriptional or translational mechanisms (Nikinmaa and Rytkönen, 2011). The increase in gene expression without post-transcriptional changes could also represent a pre-adaptation mechanism. If the concentration of contaminant increases or the duration of stress last longer, the organism would be prepared to increase the antioxidant defense activity when necessary. This adaptive capacity of *C. carpio* is confirmed by previous findings of Martinez et al. (2004) who showed the recovery of some disturbed parameters to control values in continued pollutant exposure. The differences of response between the genomic and enzymatic levels show how

important it is to analyze both responses in order to have complementary results on the occurring defense processes (Hansen et al., 2007).

At the enzymatic level, no increase was observed in the total activities of antioxidant enzymes. On the contrary, only decreasing total activities of some enzymes were recorded. The high total activities observed, especially in the liver, are sufficient to avoid oxidative stress and potential damages to *C. carpio* at these metal concentrations. The liver is the organ where most of the responses were observed, confirming that antioxidant defenses are more expressed in the liver (Atli and Canli, 2010).

The combined action of SOD and CAT is considered as the first line of defense against ROS: SOD catalyzes the dismutation of the superoxide radical into H_2O_2 and CAT reduces H_2O_2 into nontoxic H_2O and O_2. In this study, both enzymes had almost stable total activity. SOD total activity remained unchanged in the liver and the gills during the experiment. The Cu/Cd/Zn mixture used did not seem to impact this enzyme, even if previous studies with *C. carpio* recorded different responses in SOD activity according to the concentration of metal and the exposure time: it increased after 12 h exposed to 65 µg/L Cu (Eyckmans et al., 2011) or at exposure below 0.7 mg/L Cd but was inhibited by 1-2 mg/L Cd in liver (Jia et al., 2011) or after 5 days when exposed to 1.5-5 mg/L Zn^{2+} and 0.5-1.5 mg/L Pb^{2+} (Dimitrova et al., 1994). SOD activity was increased in studies with *D. labrax* (Díaz-de-Alba et al., 2017) or *S. trutta* (Hansen et al., 2006b) in presence of Cu and with *Oreochromis niloticus* exposed Zn (Abdel-Khalek et al., 2015). On the contrary, inhibition of SOD activity by Cu was recorded in *Gasterosteus aculeatus* after Cu exposure (Sanchez et al., 2005) and in *D. rerio* after Cd exposure (Pan et al., 2018). CAT activity has been reported to be stimulated by metal but this response seems to vary

488 according to the metal concentrations and the tissues involved. In our study, CAT total activity
489 decreased only at day 3 in the liver of *C. carpio* and then returned to normal, showing that the
490 mixture impact is either low or that *C. carpio* is able to adapt CAT activity really fast. However,
491 CAT activity has also been reported to be reduced by Cd exposure (from 1 µM) in *D. labrax*
492 (Roméo et al., 2000). On the contrary, CAT activity increased after 24 h in *C. carpio* exposed to
493 65 µg/L Cu (Eyckmans et al., 2011) or after 4 days in *S. trutta* exposed to a Cu-contaminated
494 river (Hansen et al., 2006b). Inhibitory response of SOD and CAT have been described as a
495 signal of contamination (Díaz-de-Alba et al., 2017; Hansen et al., 2007; Wood et al., 2011a) and
496 might be caused by a direct binding of Cu to -SH group on this enzyme (Abdel-Khalek et al.,
497 2015; Atli and Canli, 2007; Sanchez et al., 2005) or by elevated ROS concentrations due to the
498 Fenton like reaction (Atli and Canli, 2010; Eyckmans et al., 2011; Pan et al., 2018). Moreover,
499 Cd proved to induce misfolding of CAT and SOD and contributed to their reduced activities
500 (Wang et al., 2015). In our study, SOD and CAT total activities remained almost unchanged, as
501 well as the T-AOC. These activities seemed sufficient to neutralize excess ROS, as no sign of
502 oxidative stress was noticed, thus allowing *C. carpio* to avoid oxidative damage. However, metal
503 exposure induced a decrease in some enzyme total activities and this could lead to potential
504 future oxidative stress and tissue damage.
505 The total activity of enzymes involved in the glutathione system (GPx, GR and GST) decreased
506 after 3 or 7 days in liver of *C. carpio* exposed to metal mixture. The metal mixture had a direct
507 impact on the glutathione system and the antioxidative capacity of *C. carpio* may be reduced.
508 Previous studies showed the same inhibition, with a decline in GPx activity in *C. carpio* injected
509 with 10 mg/kg $CuSO_4$ (Varanka et al., 2001), in *O. niloticus* acutely and chronically exposed to
510 diverse metals (Atli and Canli, 2010), or in *D. rerio* exposed to increasing waterborne 5 and
511 25 µg/L Cd concentrations (Pan et al., 2018). Decrease in GR activity was also observed in

512 *Cottus gobio* exposed to 0.01 mg/L Cd (Dorts et al., 2012), *P. flavescens* exposed to metal
513 gradient (Giguère et al., 2005), in *O niloticus* chronically exposed to 20 µM Cd or Zn (Atli and
514 Canli, 2010) and decline in GST activity was recorded in *C. carpio* with increasing concentration
515 of Cu (Dautremepuits et al., 2004) or in *O. niloticus* acutely exposed to diverse metals (Atli and
516 Canli, 2010). However, other studies found opposite result with increase in GPx and GR activity
517 recorded in *S. trutta* exposed to Cu (Hansen et al., 2006a) and in *O. niloticus* chronically exposed
518 to Zn (Abdel-Khalek et al., 2015; Atli and Canli, 2010). These three enzymes have different roles
519 in the antioxidant defense system. GPx reduces both H_2O_2 and organic peroxides (Halliwell and
520 Gutteridge, 1999). On the other hand, GR and GST are not involved in the antioxidant defense in
521 the same way than the previous enzymes. They do not directly detoxify the cell but act in
522 combination with GSH. GR catalyses the reduction of GSH and maintains GSH/GSSG
523 homeostasis (Winston and Di Giulio, 1991), when its activity increases, more GSH is produced
524 and the antioxidant power of the cell increases. GST prevents oxidative damages by conjuging
525 breakdown products of lipid peroxides to GSH (Choi et al., 2008; Hayes and Strange, 1995).
526 Glutathione levels were not measured in our study but the decrease of these enzymes suggests a
527 reduction of its level.
528 The total activity of CAT was also reduced in *C. carpio* exposed several days to metal mixture.
529 The combined decreases of CAT and GPx total activities might become a problem at longer term.
530 If H_2O_2 is not converted into water any more (or at least not enough), oxidative stress will appear.
531

V. Conclusion

Overall, the results suggest an ability of *C. carpio* to avoid oxidative stress when exposed to a mixture of Cu, Cd and Zn at low concentrations. After a week, the levels of antioxidant defenses were still high enough to control the ROS steady-state and avoid other potential damages. However, the glutathione system was reduced at the end of the week, suggesting possible adverse health effects if the exposure was prolonged (Livingstone, 2001). But, as already described in previous study (Martinez et al., 2004) *C. carpio* has some ability to acclimate. The effect of longer exposures needs to be investigated further to fully understand how *C. carpio* cope with metal mixture.

In the field, antioxidant enzymes have been used as biomarkers to oxidative stress, because they are very sensitive indicators, even before hazardous effects appear in fish (Atli and Canli, 2010; Gul et al., 2004). Nevertheless, their variable responses (Atli and Canli, 2007; Saddick et al., 2017; Sanchez et al., 2005) make the impacts of mixtures even more difficult to interpret. Future studies need to deepen research to better understand the oxidative stress processes at gene and protein levels in fish exposed to metal pollution.

Acknowledgment

Helpful advice of Ora Johannsson is acknowledged. We thank Karin Van den Bergh and Steven Joosen for technical assistance.

Funding

This work was supported by TOP-BOF project (grant number 32252) by the University of Antwerp Research Council.

VI. Bibliography

Abdel-Khalek, A.a., Kadry, M., Hamed, A., Marie, M.-A., 2015. Ecotoxicological impacts of zinc metal in comparison to its nanoparticles in Nile tilapia; *Oreochromis niloticus*. J. Basic Appl. Zool. 72, 113-125.

Aebi, H., 1984. Catalase in vitro, Methods Enzymol. 105, 121-126.

Ahmad, H., Yousafzai, A.M., Siraj, M., Ahmad, R., Ahmad, I., Nadeem, M.S., Ahmad, W., Akbar, N., Muhammad, K., 2015. Pollution problem in River Kabul: accumulation estimates of heavy metals in native fish species. BioMed Res. Int. 537368.

Altenburger, R., Walter, H., Grote, M., 2004. What contributes to the combined effect of a complex mixture? Environ. Sci. Tech. 38, 6353-6362.

Altun, S., Özdemir, S., Arslan, H., 2017. Histopathological effects, responses of oxidative stress, inflammation, apoptosis biomarkers and alteration of gene expressions related to apoptosis, oxidative stress, and reproductive system in chlorpyrifos-exposed common carp (*Cyprinus carpio* L.). Environ. Pollut. 230, 432-443.

Andres, S., Ribeyre, F., Tourencq, J.N., Boudou, A., 2000. Interspecific comparison of cadmium and zinc contamination in the organs of four fish species along a polymetallic pollution gradient (Lot River, France). Sci. Total Environ. 248, 11-25.

Atli, G., Canli, M., 2007. Enzymatic responses to metal exposures in a freshwater fish *Oreochromis niloticus*. Comp. Biochem. Physiol. C 145, 282-287.

Atli, G., Canli, M., 2010. Response of antioxidant system of freshwater fish *Oreochromis niloticus* to acute and chronic metal (Cd, Cu, Cr, Zn, Fe) exposures. Ecotoxicol. Environ. Saf. 73, 1884-1889.

577 Banni, M., Chouchene, L., Said, K., Kerkeni, A., Messaoudi, I., 2011. Mechanisms underlying
578 the protective effect of zinc and selenium against cadmium-induced oxidative stress in zebrafish
579 *Danio rerio*. BioMetals 24, 981-992.
580 Beckman, J.S., Parks, D.A., Pearson, J.D., Marshall, P.A., Freeman, B.A., 1989. A sensitive
581 fluorometric assay for measuring xanthine dehydrogenase and oxidase in tissues. Free Radic.
582 Biol. Med 6, 607-615.
583 Beg, M.U., Al-Jandal, N., Al-Subiai, S., Karam, Q., Husain, S., Butt, S.A., Ali, A., Al-Hasan, E.,
584 Al-Dufaileej, S., Al-Husaini, M., 2015. Metallothionein, oxidative stress and trace metals in gills
585 and liver of demersal and pelagic fish species from Kuwaits' marine area. Mar. Pollut. Bull. 100,
586 662-672.
587 Benali, I., Boutiba, Z., Grandjean, D., de Alencastro, L.F., Rouane-Hacene, O., Chèvre, N., 2017.
588 Spatial distribution and biological effects of trace metals (Cu, Zn, Pb, Cd) and organic
589 micropollutants (PCBs, PAHs) in mussels *Mytilus galloprovincialis* along the Algerian west
590 coast. Mar. Pollut. Bull. 115, 539-550.
591 Bengtsson, B.E., 1974. Effect of zinc on growth of the minnow *Phoxinus phoxinus*. Oikos 25,
592 370-373.
593 Benzie, I.F., Strain, J.J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of
594 "antioxidant power": the FRAP assay. Anal. Biochem. 239, 70-76.
595 Bervoets, L., Van Campenhout, K., Reynders, H., Knapen, D., Covaci, A., Blust, R., 2009.
596 Bioaccumulation of micropollutants and biomarker responses in caged carp (*Cyprinus carpio*).
597 Ecotoxicol. Environ. Saf. 72, 720-728.
598 Burger, J., 2008. Assessment and management of risk to wildlife from cadmium. Sci. Total
599 Environ. 389, 37-45.
600 Carlberg, I., Mannervik, B., 1985. Glutathione reductase. Methods Enzymol. 113, 484-490.

601 Choi, C.Y., An, K.W., An, M.I., 2008. Molecular characterization and mRNA expression of
602 glutathione peroxidase and glutathione S-transferase during osmotic stress in olive flounder
603 (*Paralichthys olivaceus*). Comp. Biochem. Physiol. A 149, 330-337.
604 Choi, J.E., Kim, S., Ahn, J.H., Youn, P., Kang, J.S., Park, K., Yi, J., Ryu, D.-Y., 2010. Induction
605 of oxidative stress and apoptosis by silver nanoparticles in the liver of adult zebrafish. Aquat.
606 Toxicol. 100, 151-159.
607 Chu, B.X., Fan, R.F., Lin, S.Q., Yang, D.B., Wang, Z.Y., Wang, L., 2018. Interplay between
608 autophagy and apoptosis in lead(II)-induced cytotoxicity of primary rat proximal tubular cells. J.
609 Inorg. Biochem. 182, 184-193.
610 Cols Vidal, M., Hoole, D., Williams, G.T., 2008. Characterisation of cDNAs of key genes
611 involved in apoptosis in common carp (*Cyprinus carpio* L.). Fish Shellfish Immunol. 25, 494-
612 507.
613 Cortes-Diaz, M.J.A., Rodríguez-Flores, J., Castañeda-Peñalvo, G., Galar-Martínez, M., Islas-
614 Flores, H., Dublán-García, O., Gómez-Oliván, L.M., 2017. Sublethal effects induced by captopril
615 on *Cyprinus carpio* as determined by oxidative stress biomarkers. Sci. Total Environ. 605-606,
616 811-823.
617 Couture, P., Rajender Kumar, P., 2003. Impairment of metabolic capacities in copper and
618 cadmium contaminated wild yellow perch (*Perca flavescens*). Aquat. Toxicol. 64, 107-120.
619 Dautremepuits, C., Betoulle, S., Paris-Palacios, S., Vernet, G., 2004. Humoral immune factors
620 modulated by copper and chitosan in healthy or parasitised carp (*Cyprinus carpio* L.) by
621 *Ptychobothrium* sp. (Cestoda). Aquat. Toxicol. 68, 325-338.
622 Dautremepuits, C., Marcogliese, D.J., Gendron, A.D., Fournier, M., 2009. Gill and head kidney
623 antioxidant processes and innate immune system responses of yellow perch (*Perca flavescens*)

624 exposed to different contaminants in the St. Lawrence River, Canada. Sci. Total Environ. 407,
625 1055-1064.
626 De Boeck, G., De Smet, H., Blust, R., 1995a. The effect of sublethal levels of copper on oxygen
627 consumption and ammonia excretion in the common carp, *Cyprinus carpio*. Aquat. Toxicol. 32,
628 127-141.
629 De Boeck, G., Nilsson, G.E., Elofsson, U., Vlaeminck, A., Blust, R., 1995b. Brain monoamine
630 levels and energy status in common carp (*Cyprinus carpio*) after exposure to sublethal levels of
631 copper. Aquat. Toxicol. 33, 265-277.
632 Defo, M.A., Bernatchez, L., Campbell, P.G.C., Couture, P., 2015. Transcriptional and
633 biochemical markers in transplanted *Perca flavescens* to characterize cadmium- and copper-
634 induced oxidative stress in the field. Aquat. Toxicol. 162, 39-53.
635 Devasagayam, T.P., Boloor, K.K., Ramasarma, T., 2003. Methods for estimating lipid
636 peroxidation: an analysis of merits and demerits. Indian J. Biochem. Biophys. 40, 300-308.
637 Díaz-de-Alba, M., Canalejo Raya, A., Granado-Castro, M.D., Oliva Ramírez, M., El Mai, B.,
638 Córdoba García, F., Troyano-Montoro, M., Espada-Bellido, E., Torronteras Santiago, R.,
639 Galindo-Riaño, M.D., 2017. Biomarker responses of Cu-induced toxicity in European seabass
640 *Dicentrarchus labrax*: assessing oxidative stress and histopathological alterations. Mar. Pollut.
641 Bull. 124, 336-348.
642 Dimitrova, M., Tishinova, V., Velcheva, V., 1994. Combined effect of zinc and lead on the
643 hepatic superoxide dismutase-catalase system in carp (*Cyprinus carpio*). Comp. Biochem.
644 Physiol. C 108, 43-46.
645 Dorts, J., Bauwin, A., Kestemont, P., Jolly, S., Sanchez, W., Silvestre, F., 2012. Proteasome and
646 antioxidant responses in *Cottus gobio* during a combined exposure to heat stress and cadmium.
647 Comp. Biochem. Physiol. C 155, 318-324.

dos Santos Carvalho, C., Fernandes, M.N., 2008. Effect of copper on liver key enzymes of anaerobic glucose metabolism from freshwater tropical fish *Prochilodus lineatus*. Comp. Biochem. Physiol. A 151, 437-442.

Dugmonits, K., Ferencz, Á., Jancsó, Z., Juhász, R., Hermesz, E., 2013. Major distinctions in the antioxidant responses in liver and kidney of Cd^{2+}-treated common carp (*Cyprinus carpio*). Comp. Biochem. Physiol. C 158, 225-230.

Eyckmans, M., Celis, N., Horemans, N., Blust, R., De Boeck, G., 2011. Exposure to waterborne copper reveals differences in oxidative stress response in three freshwater fish species. Aquat. Toxicol. 103, 112-120.

Feng, M., He, Q., Meng, L., Zhang, X., Sun, P., Wang, Z., 2015. Evaluation of single and joint toxicity of perfluorooctane sulfonate, perfluorooctanoic acid, and copper to *Carassius auratus* using oxidative stress biomarkers. Aquat. Toxicol. 161, 108-116.

Flohé, L., Ötting, F., 1985. Superoxide dismutase assays. Methods Enzymol. 105, 93-105.

Gaete, H., Álvarez, M., Lobos, G., Soto, E., Jara-Gutiérrez, C., 2017. Assessment of oxidative stress and bioaccumulation of the metals Cu, Fe, Zn, Pb, Cd in the polychaete *Perinereis gualpensis* from estuaries of central Chile. Ecotoxicol. Environ. Saf. 145, 653-658.

Gao, D., Xu, Z.e., Zhang, X., Wang, H., Wang, Y., Min, W., 2013a. Molecular cloning, immunohistochemical localization, characterization and expression analysis of caspase-9 from the purse red common carp (*Cyprinus carpio*) exposed to cadmium. Aquat. Toxicol. 142-143, 53-62.

Gao, D., Xu, Z.e., Zhang, X., Zhu, C., Wang, Y., Min, W., 2013b. Cadmium triggers kidney cell apoptosis of purse red common carp (*Cyprinus carpio*) without caspase-8 activation. Dev. Comp. Immunol. 41, 728-737.

García-Medina, S., Galar-Martínez, M., Gómez-Oliván, L.M., Ruiz-Lara, K., Islas-Flores, H., Gasca-Pérez, E., 2017. Relationship between genotoxicity and oxidative stress induced by mercury on common carp (*Cyprinus carpio*) tissues. Aquat. Toxicol. 192, 207-215.

Giguère, A., Campbell, P.G., Hare, L., Cossu-Leguille, C., 2005. Metal bioaccumulation and oxidative stress in yellow perch (*Perca flavescens*) collected from eight lakes along a metal contamination gradient (Cd, Cu, Zn, Ni). Can. J. Fish Aquat. Sci. 62, 563-577.

Gonzalez, P., Baudrimont, M., Boudou, A., Bourdineaud, J.P., 2006. Comparative effects of direct cadmium contamination on gene expression in gills, liver, skeletal muscles and brain of the zebrafish (*Danio rerio*). Biometals 19, 225-235.

Grosell, M., Wood, C.M., 2002. Copper uptake across rainbow trout gills: mechanisms of apical entry. J. Exp. Biol. 205, 1179-1188.

Gul, S., Belge-Kurutas, E., Yildiz, E., Sahan, A., Doran, F., 2004. Pollution correlated modifications of liver antioxidant systems and histopathology of fish (*Cyprinidae*) living in Seyhan Dam Lake, Turkey. Environ. Int. 30, 605-609.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130-7139.

Halliwell, B., Gutteridge, J.M.C., 1999. Free Radicals in Biology and Medicine. 3rd ed. Oxford University Press, Oxford.

Handy, R.D., 2003. Chronic effects of copper exposure versus endocrine toxicity: two sides of the same toxicological process? Comp. Biochem. Physiol. A 135, 25-38.

Hansen, B.H., Rømma, S., Garmo, Ø.A., Olsvik, P.A., Andersen, R.A., 2006a. Antioxidative stress proteins and their gene expression in brown trout (*Salmo trutta*) from three rivers with different heavy metal levels. Comp. Biochem. Physiol. C 143, 263-274.

694 Hansen, B.H., Romma, S., Garmo, O.A., Pedersen, S.A., Olsvik, P.A., Andersen, R.A., 2007.
695 Induction and activity of oxidative stress-related proteins during waterborne Cd/Zn-exposure in
696 brown trout (*Salmo trutta*). Chemosphere 67, 2241-2249.
697 Hansen, B.H., Romma, S., Softeland, L.I., Olsvik, P.A., Andersen, R.A., 2006b. Induction and
698 activity of oxidative stress-related proteins during waterborne Cu-exposure in brown trout (*Salmo*
699 *trutta*). Chemosphere 65, 1707-1714.
700 Hayes, J.D., Strange, R.C., 1995. Potential contribution of the glutathione S-transferase
701 supergene family to resistance to oxidative stress. Free Radic. Res. 22, 193-207.
702 Hossain, S., Liu, H.-N., Nguyen, M., Shore, G., Almazan, G., 2009. Cadmium exposure induces
703 mitochondria-dependent apoptosis in oligodendrocytes. NeuroToxicol. 30, 544-554.
704 Huang, D.J., Zhang, Y.M., Song, G., Long, J., Liu, J.H., Ji, W.H., 2007. Contaminants-induced
705 oxidative damage on the carp *Cyprinus carpio* collected from the upper Yellow River, China.
706 Environ. Monit. Assess. 128, 483-488.
707 Jamers, A., Blust, R., De Coen, W., Griffin, J.L., Jones, O.A.H., 2013. An omics based
708 assessment of cadmium toxicity in the green alga *Chlamydomonas reinhardtii*. Aquat. Toxicol.
709 126, 355-364.
710 Janssens, B.J., Childress, J.J., Baguet, F., Rees, J.F., 2000. Reduced enzymatic antioxidative
711 defense in deep-sea fish. J. Exp. Biol. 203, 3717-3725.
712 Javed, M., Ahmad, I., Usmani, N., Ahmad, M., 2016. Studies on biomarkers of oxidative stress
713 and associated genotoxicity and histopathology in *Channa punctatus* from heavy metal polluted
714 canal. Chemosphere 151, 210-219.
715 Jerome, F.C., Hassan, A., Omoniyi-Esan, G.O., Odujoko, O.O., Chukwuka, A.V., 2017. Metal
716 uptake, oxidative stress and histopathological alterations in gills and hepatopancreas of
717 *Callinectes amnicola* exposed to industrial effluent. Ecotoxicol. Environ. Saf. 139, 179-193.

718 Jia, X., Zhang, H., Liu, X., 2011. Low levels of cadmium exposure induce DNA damage and
719 oxidative stress in the liver of Oujiang colored common carp *Cyprinus carpio* var. *color*. Fish
720 Physiol. Biochem. 37, 97-103.
721 Kerambrun, E., Rioult, D., Delahaut, L., Evariste, L., Pain-Devin, S., Auffret, M., Geffard, A.,
722 David, E., 2016. Variations in gene expression levels in four European zebra mussel, *Dreissena*
723 *polymorpha*, populations in relation to metal bioaccumulation: a field study. Ecotoxicol. Environ.
724 Saf. 134p1, 53-63.
725 Komjarova, I., Bury, N.R., 2014. Evidence of common cadmium and copper uptake routes in
726 zebrafish *Danio rerio*. Environ. Sci. Tech. 48, 12946-12951.
727 Kunwar, P.S., Tudorache, C., Eyckmans, M., Blust, R., De Boeck, G., 2009. Influence of food
728 ration, copper exposure and exercise on the energy metabolism of common carp (*Cyprinus*
729 *carpio*). Comp. Biochem. Physiol. C 149, 113-119.
730 Li, M., Zheng, Y., Liang, H., Zou, L., Sun, J., Zhang, Y., Qin, F., Liu, S., Wang, Z., 2013.
731 Molecular cloning and characterization of cat, gpx1 and Cu/Zn-sod genes in pengze crucian carp
732 (*Carassius auratus* var. *Pengze*) and antioxidant enzyme modulation induced by hexavalent
733 chromium in juveniles. Comp. Biochem. Physiol. C 157, 310-321.
734 Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and
735 oxidative damage in aquatic organisms. Mar. Pollut. Bull. 42, 656-666.
736 Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat.
737 Toxicol. 101, 13-30.
738 Macedo, N., J., Ferreira, T., L., 2014. Maximizing total RNA yield from TRIzol® Reagent
739 protocol: a feasibility study - Semantic Scholar, Am. Soc. Eng. Educ. 8.

740 Marie, B., Genard, B., Rees, J.-F., Zal, F., 2006. Effect of ambient oxygen concentration on
741 activities of enzymatic antioxidant defences and aerobic metabolism in the hydrothermal vent
742 worm, *Paralvinella grasslei*. Mar. Biol. 150, 273-284.
743 Martínez-Álvarez, R.M., Morales, A.E., Sanz, A., 2005. Antioxidant defenses in fish: biotic and
744 abiotic factors. Rev. Fish Biol. Fish. 15, 75-88.
745 Martinez, C.B., Nagae, M.Y., Zaia, C.T., Zaia, D.A., 2004. Acute morphological and
746 physiological effects of lead in the neotropical fish *Prochilodus lineatus*. Braz. J. Biol. 64, 797-
747 807.
748 Meng, X.-L., Li, S., Qin, C.-B., Zhu, Z.-X., Hu, W.-P., Yang, L.-P., Lu, R.-H., Li, W.-J., Nie, G.-
749 X., 2018. Intestinal microbiota and lipid metabolism responses in the common carp (*Cyprinus*
750 *carpio* L.) following copper exposure. Ecotoxicol. Environ. Saf. 160, 257-264.
751 Nikinmaa, M., Rytkönen, K.T., 2011. Functional genomics in aquatic toxicology - Do not forget
752 the function. Aquat. Toxicol. 105, 16-24.
753 Paglia, D.E., Valentine, W.N., 1967. Studies on the quantitative and qualitative characterization
754 of erythrocyte glutathione peroxidase. J. Lab. Clin. Med. 70, 158-169.
755 Pan, Y.-X., Luo, Z., Zhuo, M.-Q., Wei, C.-C., Chen, G.-H., Song, Y.-F., 2018. Oxidative stress
756 and mitochondrial dysfunction mediated Cd-induced hepatic lipid accumulation in zebrafish
757 *Danio rerio*. Aquat. Toxicol. 199, 12-20.
758 Pellegrini, M., Baldari, C.T., 2009. Apoptosis and oxidative stress-related diseases: the p66Shc
759 connection. Current Mol. Med. 9, 392-398.
760 Peterson, G.L., 1977. A simplification of the protein assay method of Lowry et al. which is more
761 generally applicable. Anal. Biochem. 83, 346-356.
762 Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT–PCR.
763 Nucleic Acids Res. 29, e45.

764 Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST©) for
765 group-wise comparison and statistical analysis of relative expression results in real-time PCR.
766 Nucleic Acids Res. 30, e36.
767 Powell, S.R., 2000. The antioxidant properties of zinc. J. Nutr. 130, 1447s-1454s.
768 Radi, A.A.R., Matkovics, B., 1988. Effects of metal ions on the antioxidant enzyme activities,
769 protein contents and lipid peroxidation of carp tissues. Comp. Biochem. Physiol. C 90, 69-72.
770 Rajeshkumar, S., Liu, Y., Ma, J., Duan, H.Y., Li, X., 2017. Effects of exposure to multiple heavy
771 metals on biochemical and histopathological alterations in common carp, *Cyprinus carpio* L. Fish
772 Shellfish Immunol. 70, 461-472.
773 Risso-de Faverney, C., Orsini, N., de Sousa, G., Rahmani, R., 2004. Cadmium-induced apoptosis
774 through the mitochondrial pathway in rainbow trout hepatocytes: involvement of oxidative stress.
775 Aquat. Toxicol. 69, 247-258.
776 Roméo, M., Bennani, N., Gnassia-Barelli, M., Lafaurie, M., Girard, J.P., 2000. Cadmium and
777 copper display different responses towards oxidative stress in the kidney of the sea bass
778 *Dicentrarchus labrax*. Aquat. Toxicol. 48, 185-194.
779 Saddick, S., Afifi, M., Abu Zinada, O.A., 2017. Effect of zinc nanoparticles on oxidative stress-
780 related genes and antioxidant enzymes activity in the brain of *Oreochromis niloticus* and *Tilapia*
781 *zillii*. Saudi J. Biol. Sci. 24, 1672-1678.
782 Sanchez, W., Palluel, O., Meunier, L., Coquery, M., Porcher, J.M., Ait-Aissa, S., 2005. Copper-
783 induced oxidative stress in three-spined stickleback: relationship with hepatic metal levels.
784 Environ. Toxicol. Pharmacol. 19, 177-183.
785 Sevcikova, M., Modra, H., Slaninova, A., Svobodova, Z., 2011. Metals as a cause of oxidative
786 stress in fish: a review. Vet. Med. 56, 537-546.

787 Sinha, A.K., Diricx, M., Chan, L.P., Liew, H.J., Kumar, V., Blust, R., De Boeck, G., 2012.
788 Expression pattern of potential biomarker genes related to growth, ion regulation and stress in
789 response to ammonia exposure, food deprivation and exercise in common carp (*Cyprinus carpio*).
790 Aquat. Toxicol. 122-123, 93-105.
791 Song, X.-B., Liu, G., Liu, F., Yan, Z.-G., Wang, Z.-Y., Liu, Z.-P., Wang, L., 2017. Autophagy
792 blockade and lysosomal membrane permeabilization contribute to lead-induced nephrotoxicity in
793 primary rat proximal tubular cells. Cell Death Disease 8, e2863.
794 Stohs, S.J., Bagchi, D., 1995. Oxidative mechanisms in the toxicity of metal ions. Free Radic.
795 Biol. Med. 18, 321-336.
796 Tripathi, S., Mishra, B.B., Tripathi, S.P., 2012. Impact of zinc sulphate on biochemical
797 parameters in reproductive cycle of *Colisa fasciatus*. Int. J. Basic Appl. Sci. 1, 2277-1921.
798 Tybout, A., Sternthal, B., 2001. Can I test for simple effects in the presence of an insignificant
799 interaction? . J. Consum. Psychol. 10, 5-10.
800 USEPA, 2002. Short-term methods for estimating the chronic toxicity of effluents and receiving
801 waters to freshwater organisms. 4th ed. U.S. Environmental Protection Agency, Washington, DC,
802 USA.
803 Varanka, Z., Rojik, I., Varanka, I., Nemcsók, J., Ábrahám, M., 2001. Biochemical and
804 morphological changes in carp (*Cyprinus carpio* L.) liver following exposure to copper sulfate
805 and tannic acid. Comp. Biochem. Physiol. C 128, 467-477.
806 VMM, 2014. http://geoloket.vmm.be/Geoviews/ (consulted October 2018)
807 Wang, J., Zhang, H., Zhang, T., Zhang, R., Liu, R., Chen, Y., 2015. Molecular mechanism on
808 cadmium-induced activity changes of catalase and superoxide dismutase. Int. J. Biol. Macromol.
809 77, 59-67.

Wang, S., Zheng, S., Zhang, Q., Yang, Z., Yin, K., Xu, S., 2018. Atrazine hinders PMA-induced neutrophil extracellular traps in carp via the promotion of apoptosis and inhibition of ROS burst, autophagy and glycolysis. Environ. Pollut. 243, 282-291.

Wang, S., Zhang, Q., Zheng, S., Chen, M., Zhao, F., Xu, S., 2019. Atrazine exposure triggers common carp neutrophil apoptosis via the CYP450s/ROS pathway. Fish Shellfish Immunol. 84, 551-557.

Wang, Y., Fang, J., Leonard, S.S., Krishna Rao, K.M., 2004. Cadmium inhibits the electron transfer chain and induces reactive oxygen species. Free Radic. Biol. Med. 36, 1434-1443.

Weydert, C.J., Cullen, J.J., 2010. Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. Nat. Protoc. 5, 51-66.

Winston, G.W., Di Giulio, R.T., 1991. Prooxidant and antioxidant mechanisms in aquatic organisms. Aquat. Toxicol. 19, 137-161.

Wood, C.M., Farrell, A.P., Brauner, C.J., 2011a. Homeostasis and toxicology of essential metals. In: Farrell, A.P., Brauner, C.J. (Eds.), Fish Physiology, vol. 31A. Academic Press, Elsevier, Amsterdam, 520 pp.

Wood, C.M., Farrell, A.P., Brauner, C.J., 2011b. Homeostasis and toxicology of non-essential metals. In: Farrell, A.P., Brauner, C.J. (Eds.), Fish Physiology, vol. 31B. Academic Press, Elsevier, Amsterdam, 528 pp.

Wu, P., Jiang, W.-D., Liu, Y., Chen, G.-F., Jiang, J., Li, S.-H., Feng, L., Zhou, X.-Q., 2014. Effect of choline on antioxidant defenses and gene expressions of Nrf2 signaling molecule in the spleen and head kidney of juvenile Jian carp (*Cyprinus carpio* var. *Jian*). Fish Shellfish Immunol. 38, 374-382.

Zhang, Z., Zheng, Z., Cai, J., Liu, Q., Yang, J., Gong, Y., Wu, M., Shen, Q., Xu, S., 2017. Effect of cadmium on oxidative stress and immune function of common carp (*Cyprinus carpio* L.) by transcriptome analysis. Aquat. Toxicol. 192, 171-177.

Zheng, G.-H., Liu, C.-M., Sun, J.-M., Feng, Z.-J., Cheng, C., 2014. Nickel-induced oxidative stress and apoptosis in *Carassius auratus* liver by JNK pathway. Aquat. Toxicol. 147, 105-111.

Zheng, J.-L., Yuan, S.-S., Wu, C.-W., Li, W.-Y., 2016. Chronic waterborne zinc and cadmium exposures induced different responses towards oxidative stress in the liver of zebrafish. Aquat. Toxicol. 177, 261-268.

841 Table 1: Set of primers (f = forward; R = reverse) designed for *Cyprinus carpio* using Primer
842 blast (NCBI) and used for gene expression analysis by quantitative RT-PCR. Reference gene:
843 elongation factor 1α; target genes: superoxide dismutase Cu-Zn (SOD), catalase (CAT,
844 glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferase (GST) and
845 caspase 9 (CASP)

Gene	accession number	primer 5'--> 3'	Tm °C	% GC	amplicon length	% efficiency
EF1α	**AF485331.1**	F - TGGAGATGCTGCCATTGT	60.1	50,0	178	96.3
		R - TGCAGACTTCGTGACCTT	60.2	50,0		
SOD Cu-Zn	**XM_019111694.1**	F - CTGTGTGGGCACTGTCTTCTT	60.5	52.4	100	95.3
		R - GACACACACACATCCTGTCCG	61.2	57.1		
CAT	**GQ376154.1**	F - CCCTCTGATTCCTGTGGGAC	59.5	60,0	172	105.3
		R - CCGATGCCTATGTGTGTCCG	60.9	60,0		
GPx	**GQ376155.1**	F - CGTCGCTTTGAGGCACAAC	60.1	57.9	125	100.3
		R - GGCATTCTCCTGATGTCCGAA	60.1	52.4		
GR	**JF411607.1**	F - GAGAAGTACGACACCATCCA	60,0	50,0	52	94.6
		R - CACACCTATTGAACTGAGATTGAG	48.9	41.7		
GST	**DQ411314.1**	F - ACCCTGAACACACCAGCAAC	60.8	55,0	180	83.0
		R - GAGTTCACAAATAAAGCGGCCC	60.4	50,0		
CASP	**KC676314.1**	F - TTGAGGAGAATGCTGCCACG	60.7	55,0	176	94.3
		R - TCCCACTGCAGCAAAAAGTG	59.3	50,0		

846

Table 2: Total enzymatic activity (U/g of wet tissue) of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione S-transferase in liver and gills of *Cyprinus carpio* exposed to Cu/Cd/Zn mixture for 1, 3 or 7 days (mean ± standard error, N = sample size; (N) (in parenthese) = sample size when N was different from the treatment). Asterisks indicate a significant difference between the control and the exposed group for the same day (*p < 0.05 and **p < 0.01); different letters indicate significant difference between the groups exposed for different duration, within the same condition (p < 0.05).

	Enzyme	Day 1		Day 3		Day 7	
		Control	Exposed	Control	Exposed	Control	Exposed
N		10	10	10	10	10	10
Mass		3.38 ± 0.32	3.25 ± 0.25	3.59 ± 0.41	3.73 ± 0.42	4.12 ± 0.41	3.64 ± 0.57
Length		62.94 ± 1.82	63.2 ± 1.53	64.75 ± 2.23	65.99 ± 2.61	68.15 ± 2.34	66.1 ± 3.33
Liver	SOD	5005 ± 286	5418 ± 632 (9)	5057 ± 547 (8)	5540 ± 455 (9)	5829 ± 608	5453 ± 826 (8)
	CAT	5749 ± 517	5643 ± 776 (9)	7347 ± 873 (9)	4905 ± 399*	7288 ± 712	6054 ± 611
	GPx	8566 ± 901	8169 ± 685	10612 ± 1091	8008 ± 494	10208 ± 588	5044 ± 986*
	GR	1195 ± 117	1172 ± 130^{a}	1260 ± 137	865.5 ± 77.3*^{ab}	1064 ± 68.3	674 ± 85.6**^{b}
	GST	0.27 ± 0.02	0.28 ± 0.03	0.33 ± 0.04	0.26 ± 0.02	0.35 ± 0.02	0.25 ± 0.03*
Gills	SOD	4232 ± 614 (8)	4034 ± 526^{a}	4095 ± 385 (9)	4047 ± 489^{a}	2918 ± 583 (9)	2118 ± 458^{b}
	CAT	172 ± 36 (8)	197.1 ± 35.53 (8)	159.8 ± 19.2	151.2 ± 32.1	194 ± 37	131.4 ± 18.3 (9)
	GPx	1227 ± 278 (9)	1152 ± 152.9^{ab}	994.1 ± 159	1379 ± 223^{a}	598.4 ± 55.3	595.6 ± 128^{b}
	GR	593.6 ± 33.9^{AB}	647.9 ± 23.6 (9)	638.5 ± 25.3^{A}	657.6 ± 29.1	530 ± 22.7^{B}	563.2 ± 48.8
	GST	0.11 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.11 ± 0.01 (9)	0.1 ± 0.01

Table 3: Relative expression of superoxide dismutase, catalase glutathione peroxidase, glutathione reductase and glutathione S-transferase mRNA in liver and gills of *Cyprinus carpio* exposed to Cu/Cd/Zn mixture for 1, 3 or 7 days (mean ± standard error, N = sample size; (N) (in parenthese) = sample size when N was different from the treatment). Asterisks indicate a significant difference between the control and the exposed group for the same day (*p < 0.05 and **p < 0.01); different letters indicate significant difference between the groups exposed for different duration, within the same condition (p < 0.05).

	Gene	Day 1		Day 3		Day 7	
		Control	Exposed	Control	Exposed	Control	Exposed
N		10	10	10	10	10	10
Mass		3.38 ± 0.32	3.25 ± 0.25	3.59 ± 0.41	3.73 ± 0.42	4.12 ± 0.41	3.64 ± 0.57
Length		62.94 ± 1.82	63.2 ± 1.53	64.75 ± 2.23	65.99 ± 2.61	68.15 ± 2.34	66.1 ± 3.33
Liver	SOD	1.07 ± 0.12 (9)	0.91 ± 0.15 (9)	1.17 ± 0.24 (9)	0.99 ± 0.18	1.17 ± 0.21	1.28 ± 0.2 (8)
	CAT	1.05 ± 0.11 (9)	0.64 ± 0.08[a]	1.19 ± 0.21 (9)	1.52 ± 0.32[a]	1.69 ± 0.26	2.67 ± 0.3[b] (8)
	GPx	1.08 ± 0.15 (9)	0.86 ± 0.1[a]	1.58 ± 0.38	2.01 ± 0.33[b] (9)	1.45 ± 0.32 (9)	1.86 ± 0.28[b] (9)
	GR	1.09 ± 0.18 (9)	0.64 ± 0.18[a]	0.91 ± 0.16 (9)	1.34 ± 0.27[ab] (9)	1.36 ± 0.33	2.08 ± 0.29[b] (8)
	GST	1.05 ± 0.1 (9)	0.56 ± 0.1[a]	2.46 ± 0.76 (7)	3.21 ± 0.86[b]	1.99 ± 0.55	2.33 ± 0.45[b] (9)
Gills	SOD	0.94 ± 0.19 (9)	1.01 ± 0.22	1.05 ± 0.11	1.54 ± 0.41 (9)	1.11 ± 0.19	1.07 ± 0.26 (9)
	CAT	1.19 ± 0.36 (9)	1.18 ± 0.19	1.03 ± 0.08	2.25 ± 0.4** (9)	1.07 ± 0.12	2.13 ± 0.39* (9)
	GPx	1.12 ± 0.33 (9)	1.5 ± 0.36	1.11 ± 0.19	1.85 ± 0.52 (9)	1.15 ± 0.19	1.78 ± 0.33 (9)
	GR	1.1 ± 0.34 (9)	1.88 ± 0.42	1.02 ± 0.08	2.46 ± 0.53** (9)	1.04 ± 0.1	1.86 ± 0.54 (9)
	GST	0.85 ± 0.3 (8)	0.92 ± 0.18	1.07 ± 0.12	0.98 ± 0.14	1.14 ± 0.16	0.56 ± 0.09 (9)

Figure captions:

Figure 1: General experimental schema for the one-week sublethal exposure of *Cyprinus carpio* to tertiary Cu/Cd/Zn metal mixture

Figure 2: Protein concentration (mg/g of wet tissue) in liver (A) and gills (B) of *Cyprinus carpio* exposed to Cu/Cd/Zn mixture for 1, 3 or 7 days (mean ± standard error, n = 10). Asterisks indicate a significant difference between the control and the exposed group for the same day (*$p < 0.05$ and **$p < 0.01$); different letters indicate significant difference between the groups exposed for different duration, within the same condition ($p < 0.05$).

Figure 3: Relative expression of caspase 9 mRNA in liver (A) and gills (B) of *Cyprinus carpio* exposed to Cu/Cd/Zn mixture for 1, 3 or 7 days (mean ± standard error). Asterisks indicate a significant difference between the control and the exposed group for the same day (*$p < 0.05$); different letters indicate significant difference between the groups exposed for different duration, within the same condition ($p < 0.05$).

Figure 4: Total enzymatic activity (U/g of wet tissue) of xanthine oxidase (XO) and level of thiobarbituric substances (TBARS, nmol/g of wet tissue) in liver (A) and gills (B) of *Cyprinus carpio* exposed to Cu/Cd/Zn mixture for 1, 3 or 7 days (mean ± standard error). Different letters indicate significant difference between the groups exposed for different duration, within the same condition ($p < 0.05$).

882 Figure 5: Total antioxidative capacity expressed as Trolox equivalent (µmol/g of wet tissue) in
883 liver (A) and gills (B) of *Cyprinus carpio* exposed to Cu/Cd/Zn mixture for 1, 3 or 7 days (mean
884 ± standard error).