Antagonistic bioaccumulation of waterborne Cu(II) and Cd(II) in common carp (Cyprinus carpio) and effects on ion-homeostasis and defensive mechanisms

Aquatic Toxicology

Antagonistic bioaccumulation of waterborne Cu(II) and Cd(II) in common carp (Cyprinus carpio) and effects on ion-homeostasis and defensive mechanisms.

--Manuscript Draft--

Manuscript Number:	AQTOX_2020_87R1
Article Type:	Research Paper
Keywords:	mixture stress; metal pollution; defense mechanisms; ionoregulation; Cyprinus carpio.
Corresponding Author:	Giovanni Castaldo University of Antwerp Antwerp, Belgium
First Author:	Giovanni Castaldo
Order of Authors:	Giovanni Castaldo
	Gunter Flipkens
	Marion PILLET
	Raewyn Town
	Lieven Bervoets
	Ronny Blust
	Gudrun De Boeck
Abstract:	In the aquatic environment, metals are present as mixtures, therefore studies on mixture toxicity are crucial to thoroughly understand their toxic effects on aquatic organisms. Common carp were used to assess the effects of short-term Cu(II) and Cd(II) mixtures, using a fixed concentration of one of the metals, representing 25 % of its individual 96h-LC 50 (concentration lethal for 50 % of the population) combined with a variable concentration of the other metal corresponding to 10, 25 or 50 % of its 96h-LC 50 , and vice versa. Our results showed a fast Cu and Cd bioaccumulation, with the percentage of increase in the order gill > liver > carcass. An inhibitory effect of Cu on Cd uptake was observed; higher Cu concentrations at fixed Cd levels resulted in a decreased accumulation of Cd. The presence of the two metal ions resulted in losses of total Na, K and Ca. Fish tried to compensate for the Na loss through the induction of the genes coding for Na + /K + -ATPase and H + -ATPase. Additionally, a counterintuitive induction of the gene encoding the high affinity copper transporter (CTR1) occurred, while a downregulation was expected to prevent further metal ion uptake. An induction of defensive mechanisms, both metal ion binding protein and anti-oxidant defences, was observed. Despite the metal accumulation and electrolyte loss, the low mortality suggest that common carp is able to cope with these metal levels, at least during a one-week exposure.

- Common carp were exposed to several binary mixtures of copper (Cu) and cadmium (Cd) at fixed and variable concentrations.
- Copper and Cd are quickly accumulated in the gills, even though an antagonistic-like effect on cadmium uptake, caused by Cu was shown.
- The metal mixtures led to a drop in sodium (Na) levels already after one day of exposure, whereas calcium (Ca) levels started to decrease only by the end of the experiment.
- Induction of defensive mechanisms, such as metallothionein (MT) and glutathione reductase (GR), in order to cope with the increasing amount of metals.

Antagonistic bioaccumulation of waterborne Cu(II) and Cd(II) in common carp (*Cyprinus carpio*) and effects on ion-homeostasis and defensive mechanisms.

Castaldo, G. *[a]; Flipkens, G.[a]; Pillet, M.; Town, R. M.; Bervoets, L.; Blust, R.; and De Boeck, G.

Systemic Physiological and Ecotoxicological Research (SPHERE), Department of Biology, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerp, Belgium.

[a]CG and FG contributed equally to this paper.

*Corresponding author at: Systemic Physiological and Ecotoxicological Research (SPHERE), Department of Biology, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerp, Belgium. E-mail address: Giovanni.Castaldo@uantwerpen.be

Abstract

In the aquatic environment, metals are present as mixtures, therefore studies on mixture toxicity are crucial to thoroughly understand their toxic effects on aquatic organisms. Common carp were used to assess the effects of short-term Cu(II) and Cd(II) mixtures, using a fixed concentration of one of the metals, representing 25 % of its individual 96h-LC_{50} (concentration lethal for 50 % of the population) combined with a variable concentration of the other metal corresponding to 10, 25 or 50 % of its 96h-LC_{50}, and vice versa. Our results showed a fast Cu and Cd bioaccumulation, with the percentage of increase in the order gill > liver > carcass. An inhibitory effect of Cu on Cd uptake was observed; higher Cu concentrations at fixed Cd levels resulted in a decreased accumulation of Cd. The presence of the two metal ions resulted in losses of total Na, K and Ca. Fish tried to compensate for the Na loss through the induction of the genes coding for Na^+/K^+-ATPase and H^+-ATPase. Additionally, a counterintuitive induction of the gene encoding the high affinity copper transporter (CTR1) occurred, while a downregulation was expected to prevent further metal ion uptake. An induction of defensive mechanisms, both metal ion binding protein and anti-oxidant defences, was observed. Despite the metal accumulation and electrolyte loss, the low mortality suggest that common carp is able to cope with these metal levels, at least during a one-week exposure.

Keywords: mixture stress, metal pollution, defense mechanisms, ionoregulation, *Cyprinus carpio*.

1. Introduction

The aquatic environment is the main sink of pollutants produced by industries, sewage, agriculture and mining activities. Trace metals are persistent, non-degradable pollutants that can accumulate in the aquatic food web (Díaz-de-Alba et al. 2017). Two groups of metals can be distinguished, namely essential and non-essential. Metal ions belonging to the first group are important for many biological processes but can pose a risk for the organism at concentrations that are too low or too high. Essential metal ions such as those of zinc (Zn), copper (Cu) and iron (Fe) have a well-known role in animal cells. For example, Cu serves as a catalytic cofactor in several enzymes, thus it is essential in cellular respiration, connective tissue formation, melanin production and so on (Zhao et al. 2014). Copper ion uptake can occur through the high-affinity Cu transporter (CTR1) or by the divalent metal ion transporter (DMT1) (Mackenzie et al. 2004, Sevcikova et al. 2011). Furthermore, it can be taken up through a putative apical sodium (Na^+)-channel, leading to competition with Na^+ at the uptake site. Moreover, the uptake can be facilitated by exchangers, such as the sodium-hydrogen exchangers (NHEs), located in the branchial epithelial cells, through the extrusion of H^+ (Grosell 2011, Niyogi et al. 2015). Elevated Cu ion levels can be dangerous for aquatic organisms, since they can lead to a disturbance in acid-base balance (Grosell 2011), alter Na^+ homeostasis by decreasing the activity of the Na^+/K^+-ATPase and damage the cells (De Boeck et al. 2001). Moreover, Cu ions can promote oxidative stress (Bopp et al. 2008).

Non-essential metal species such as those of mercury (Hg), lead (Pb) and cadmium (Cd) have no known biological function in vertebrates and are toxic even at low concentrations (Danabas et al. 2018). Cd^{2+} is considered a threat for animals because it can alter ionoregulation, modulate protein structure and generate oxidative stress (Ferain et al. 2018). Toxic effects induced by Cd can result in hypocalcaemia, as a result of the competition between calcium (Ca^{2+}) and Cd^{2+} (Cinier et al. 1997, McGeer et al. 2011). Moreover, Cd can cause changes in superoxide dismutase (SOD) and glutathione (GSH) activity in fish liver (Jia et al. 2011). Ultimately, chronic waterborne Cd exposure can induce immunosuppression in common carp leading to death (Zhang et al. 2017).

The antioxidant defence system plays a crucial role in preventing deleterious effects caused by reactive oxygen species (ROS). Enzymes such as SOD, and catalase (CAT) represent the first line of defence, converting superoxide ($O_2^{-\bullet}$) into hydrogen peroxide (H_2O_2), and H_2O_2 into water (H_2O) and oxygen (O_2) (Pillet et al. 2019). Furthermore, GSH plays an important role in ROS defence and as a chelating agent for metal ions (Lange et al. 2002). Thus, the presence of enzymes such glutathione reductase (GR) and glutathione-S-transferase (GST) is needed for glutathione metabolism. Glutathione-S-transferase metabolizes lipid hydroperoxides (Dautremepuits et al. 2009) and mediates the conjugation reaction of GSH with electrophilic compounds, causing the depletion of GSH (Dickinson and Forman 2002). Glutathione reductase catalyses the reduction of glutathione disulfide (GSSG) in order to maintain a constant ratio of GSH/GSSG (Couto et al. 2016). In addition to the antioxidant system, metallothioneins (MT) play an important role in protecting the organism from metal toxicity. Metallothioneins are cysteine rich proteins, that play a significant role in essential metal ion homeostasis (e.g. Zn and Cu) and binding of non-essential metal ions (e.g. Cd) for sequestration (Atli and Canli 2008, Jakimska et al. 2011). Their levels and activity in tissues can be stimulated by both essential and non-essential metal ions (Hogstrand and Haux 1990, Wu et al. 1999).

Organisms in aquatic ecosystems are generally exposed to a mixture of metals that can be taken up via common uptake routes and interact with each other during uptake (Komjarova and Blust 2009). This interaction can stimulate or inhibit the uptake of particular compounds. For example, a non-competitive interaction can occur between Cu and silver (Ag) in which Ag can stimulate gill-Cu binding but not the other way around (Niyogi et al. 2015). A competitive interaction on the other hand can occur between Cd^{2+} and Zn^{2+}, since they have a comparable electron configuration and they both have a high affinity for thiol groups (Brzóska and

Moniuszko-Jakoniuk 2001). Cadmium uptake can also be inhibited in presence of Cu as demonstrated in several organisms, such as zebrafish, rainbow trout and freshwater mussel (*Pyganodon grandis*) (Stewart 1999, Franklin et al. 2002, Kamunde and MacPhail 2011b, Komjarova and Bury 2014). Deleterious effects of mixtures of these metal ions have already been studied in different model species such as Mediterranean mussel (*Mytilus galloprovincialis*) (Benali et al. 2017), rainbow trout (*Oncorhynchus mykiss*) (Kamunde and MacPhail 2011a) and zebrafish (*Danio rerio*) (Komjarova and Bury 2014).

The aim of the present study was to investigate bioaccumulation, ionoregulation and responses of defensive mechanisms in common carp after a short-term exposure to waterborne binary mixtures of Cu(II) and Cd(II), using environmentally relevant concentrations. In addition, possible interactions between the two metal ions were investigated. Therefore, common carp were exposed to a series of sublethal mixtures of Cu (nominal concentrations used: 0.07, 0.19 and 0.38 µM) and Cd (nominal concentrations used: 0.026, 0.07, 0.13 µM) using environmentally relevant concentrations. In Flanders (Belgium) where this study was conducted, the water quality guideline for dissolved Cu in surface water is set to 0.11 µM, whereas for Cd the maximum values ranged between 0.004 to 0.013 µM depending on water hardness (Belgian Official Journal, 2015). However, in reality these limits are often exceeded. For instance, in Flanders the Flemish Environmental Agency (VMM) has measured concentration up to 2.05 µM for Cu and 1.06 µM for Cd (VMM 2016). Furthermore, a field study done in Flanders over 14 different locations reported dissolved metal concentrations up to 0.4 µM and 0.2 µM for Cu and Cd, respectively (Bervoets and Blust 2003). Therefore, the metal concentrations used in this study can be considered as environmentally relevant.

With this work, together with all the previous studies investigating and showing the complexity of metal mixture scenarios (Komjarova and Blust 2009, Niyogi et al. 2015, Brix et al. 2017, Pillet et al. 2019) we aim to provide new insights into understanding metal accumulation and toxicity in multi-metal exposure scenarios. We hypothesize that metal mixtures would remain sub-lethal, as our exposures were relatively short and maximum exposure concentrations were 25 % + 50 % of the 96h-LC_{50}. Furthermore, we anticipate a quick metal bioaccumulation for both Cu and Cd, even though a reduced accumulation of Cd is expected in presence of Cu. Moreover, we expect that metal accumulation would trigger defensive mechanisms, such as MT and GR to mitigate possible deleterious effects. Regarding ion-homeostasis, in agreement with previous results from our lab, we expect a Na but not a Ca loss (Castaldo et al. 2020, Delahaut et al. 2020)

2. Material and methods

2.1. Experimental animals

Juvenile common carp were obtained from the fish hatchery at Wageningen University, the Netherlands. The fish were kept for several months at the University of Antwerp in a 1000 l aquarium filled with tap water before the experiments started. Fish were fed once a day *ad libitum* with commercial fish food (Hikari® Staple™, Klundert, The Netherlands). Temperature was kept at 20 °C, oxygen was provided with air stones and the photoperiod was set to 12 h light and 12 h dark (12L:12D). A biofilter was provided to maintain water quality. Three weeks before the start of each experiment, 200 fish were transferred and divided over two 200 l polyethylene tanks (100 fish per tank) filled with EPA medium-hard water (Weber 1991). Artificial medium hard water was reconstituted using four salts $NaHCO_3$ (1.14 mM); $CaSO_4 \cdot 2H_2O$ (0.35 mM); $MgSO_4 \cdot 7H_2O$ (0.5 mM) and KCl (0.05 mM) (VWR Chemicals). Oxygen was provided with air stones and the photoperiod was set to 12L:12D. Experimental methods complied with regulations of the Federation of European Laboratory Animal Science Associations (FELASA) and were approved by the local ethics committee of the University of Antwerp (Permit Number: 2015-94, Project 32252).

2.2. Experimental set-up

Two series of one-week waterborne exposures to binary mixtures of Cd and Cu were performed on common carp (length = 58.5 ± 6.8 mm; weight = 2.3 ± 0.9 g mean ± standard deviation (SD)). Besides control groups, treatments consisted of a fixed concentration of one of the metals at 25 % of the 96h-LC_{50} previously calculated in our lab (Delahaut et al. 2020) combined with 10, 25 and 50 % of the 96h-LC_{50} of the other metal (indicated as Cu_{fix}/Cd_{var} or Cd_{fix}/Cu_{var}). Exposure tanks consisting of five (plus one as backup in case of mortality) double-walled polypropylene (PP) containers per treatment, each filled with 9 l of EPA medium-hard water and containing six fish, were set up in the climate chamber at 20°C. In each container oxygen was provided with an air stone. In order to avoid the accumulation of waste products, such as ammonia, 90 % of the water was changed daily. As indicated in Castaldo et al. (2020), aerated EPA medium-hard water was prepared 24 h in advance and kept at 20 °C. Conductivity (275 ± 6.2 µS/cm) and pH (8.2 ± 0.2) were measured daily. Water samples were collected before (N = 120) and after (N = 140) the water change to check stability of the total metal concentrations. The nominal and measured metal concentrations in the water for both exposure series are shown in Table 1 and 2. Metal speciation was calculated with VMinteq (Supplementary information, SI-Table 1 and SI-Table 2).

Table 1: Metal concentrations (mean ± SD) used for the different treatments in the exposure series Cu_{fix}/Cd_{var}. If the measured concentrations were below the minimum quantification limit of the instrument (BMQL), this was added together with the quantification limit.

	Nominal concentration	**Measured concentration**
Control	0 µM Cu 0 µM Cd	0.0031 ± 0.0031 µM Cu < 0.00089 µM (BMQL) Cd
Treatment Cu_{fix}/Cd_{10}	25 % LC_{50} Cu (0.19 µM) 10 % LC_{50} Cd (0.026 µM)	0.15 ± 0.020 µM Cu 0.025 ± 0.0027 µM Cd
Treatment Cu_{fix}/Cd_{25}	25 % LC_{50} Cu (0.19 µM) 25 % LC_{50} Cd (0.065 µM)	0.16 ± 0.020 µM Cu 0.062 ± 0.0062 µM Cd
Treatment Cu_{fix}/Cd_{50}	25 % LC_{50} Cu (0.19 µM) 50 % LC_{50} Cd (0.13 µM)	0.16 ± 0.024 µM Cu 0.12 ± 0.012 µM Cd

Table 2: Metal concentrations (mean ± SD) used for the different treatments in the exposure series Cd_{fix}/Cu_{var}. If the measured concentrations were below the minimum quantification limit of the instrument (BMQL), this was added together with the quantification limit.

	Nominal concentration	**Measured concentration**

Control	0 µM Cu 0 µM Cd	0.0047 ± 0.0013 µM Cu < 0.00089 µM (BMQL) Cd
Treatment Cd_{fix}/Cu_{10}	10 % LC_{50} Cu (0.077 µM) 25 % LC_{50} Cd (0.065 µM)	0.068 ± 0.011 µM Cu 0.060 ± 0.0062 µM Cd
Treatment Cd_{fix}/Cu_{25}	25 % LC_{50} Cu (0.19 µM) 25 % LC_{50} Cd (0.065 µM)	0.16 ± 0.020 µM Cu 0.062 ± 0.0062 µM Cd
Treatment Cd_{fix}/Cu_{50}	50 % LC_{50} Cu (0.38 µM) 25 % LC_{50} Cd (0.065 µM)	0.34 ± 0.036 µM Cu 0.061 ± 0.0062 µM Cd

2.3. Metal bioaccumulation and electrolyte levels

At each sampling point (day one, three and seven) 10 fish per treatment (two from each container), were euthanized using an overdose of MS-222 (pH 7.0, ethyl 3-aminobenzoate methane-sulfonic acid, 400 mg/l, Acros Organics, Geel, Belgium). Muscle samples were cut near the caudal fin from individual fish and the following tissues were pooled from two fish in order to obtain enough tissue for ion and metal analysis: the first and the fourth gill arches from both the left and the right side as well as the brain and the liver. The samples were collected in pre-weighed Eppendorf tubes. The remaining carcasses from two fish were pooled and collected in pre-weighed 50 ml Falcon tubes to estimate the whole body accumulation. The samples were stored at -80 °C.

Metal and electrolyte content were determined in five samples of each tissue, at each sampling point. Reference material (SRM-2976, mussel tissue, National Institute of Standards and Technology, Gaithersburg, MD, USA), collected in pre-weighed Eppendorf tubes was included in the analysis as a quality control. The samples and the reference material were dried for 48 h, and the dry weight (dw) was recorded using a precision scale (Sartorius SE2, ultra-microbalance). Briefly, the digestion process (Blust et al. 1988, Reynders et al. 2006a) consisted of 12 h digestion at room temperature using 69 % concentrated HNO_3, followed by three microwave steps. Afterwards, H_2O_2 was added, to destroy the fat tissue, followed by another microwave digestion. Carcass samples, collected in pre-weighed 50 ml Falcon tubes were processed similarly. The samples, after an initial digestion step with 69 % HNO_3 at room temperature for 12 h, were digested using a hot block (Environmental Express, Charleston, SC, USA) for 30 min at 100 °C. At the end of the digestion process, all the samples were diluted using ultrapure Milli-Q (MQ), to reach a final acid volume concentration between 1 and 3 %. Metal and electrolyte content were determined respectively using a 7700x ICP-MS (Agilent Technologies, Santa Clara, CA, USA) and an iCAP 6300 Duo (Thermo Scientific, Waltham, MA, USA). Results obtained with ICP-MS and iCAP refer to the total element content (e.g total Cu, Na). Therefore, charges were only added when relevant for the discussion.

2.4. RNA extraction and real time PCR

The second and the third gill arch of individual fish and an aliquot of the pooled liver samples of two fish were used for RNA extraction and gene expression. Total RNA was extracted from samples (~ 20/30 mg) using Trizol (Invitrogen, Merelbeke, Belgium) following the manufacturer's instructions. Nano-Drop spectrophotometry (NanoDrop Technologies, Wilmington, DE) was used to determine RNA quantity and quality, whereas integrity was evaluated with a 1% agarose gel with ethidium bromide (500 µg/ml). DNase treatment was performed using the commercial RNase free kit DNase I from Thermo Fisher Scientific (Waltham, MA, USA). Then 1 µg of RNA was transcribed to cDNA according to RevertAid H minus First strand cDNA synthesis kit protocol (Thermo fisher, Fermentas, Cambridgeshire). According to the OD260/OD280 nm absorption ratio (higher than 1.8), four samples were selected and used for qPCR. Real-time PCR was performed using a Mx3000P QPCR System (Agilent Technologies, Belgium). The assay was performed in duplicate in a final reaction volume of 20 µl containing 10 µl of Brilliant III Ultra-Fast QPCR Master Mix (Agilent), 500 nM of each primer (reverse and forward), 5.7 µl of sterile water, 0.3 µl of reference dye and 5 ng of cDNA. PCR amplification was carried out following the Brilliant III Ultra-Fast QPCR Master

Mix (Agilent) protocol for Agilent Mx3000P QPCR system. Oligonucleotides primers were taken from literature: elongation factor 1α (eEF) (Sinha et al. 2012), β-actin (Wu et al. 2014); H^+-ATPase (Sinha et al. 2016), catalase (CAT) (Wu et al. 2014), superoxide dismutase Cu-Zn (SOD) (Wu et al. 2014), glutathione reductase (GR) (Wu et al. 2014), glutathione S-transferase (GST) (Casatta et al. 2017), metallothionein (MT) (Reynders et al. 2006b), Na^+/H^+-exchanger (NHE-2) (Castaldo et al. 2020) and Na^+/K^+-ATPase (Castaldo et al. 2020). Primers for CTR1 were designed using NCBI resources Primer blast and synthesized as highly purified salt-free "OliGold" primers by Eurogentec (Eurogentec, Seraing, Belgium). Primer sequences, annealing temperature and primer efficiency are given in SI-Table 10. Primer efficiency was determined based on the slope of the standard curve, using a serial dilution of cDNA.

2.5. Statistical analysis

All data were presented as mean values ± S.D. For the statistical analyses, normality of the data was tested with the Shapiro-Wilk test. Two-way analyses of variance (ANOVA) were performed on all accumulation and gene expression data, followed by Tukey test. Data were considered statistically significant when p-value < 0.05. All statistical tests were performed with GraphPad Prism version 8.02 for Windows (GraphPad Software, La Jolla California USA). According to Custer et al. (2000), for metal concentrations below the minimum quantification limit (BMQL), a value of MQL/2 was assigned. If > 50% of the observations were BMQL, no statistical tests were conducted. Data presented in the supplementary information, such as curve fitting the metal bioaccumulation (non-linear, Michaelis-Menten, one-phase decay) and sodium loss (two-phase decay) as a function of time and exposure concentration, were analysed using the same software.

3. Results

3.1. Dynamics of Cu and Cd bioaccumulation

3.1.1. Copper bioaccumulation

Copper accumulation in gill tissue showed a similar pattern for both the exposure scenarios (Fig. 1.1A and 1.2A). In general Cu is always higher in the treatment compared with the control. This increase seems relatively independent from waterborne Cu concentrations. However, at day seven fish in treatment Cd_{fix}/Cu_{25} accumulated more Cu in comparison with treatment Cd_{fix}/Cu_{10}. After seven days of exposure, a strong increase in Cu was observed in all the treatments, compared with the previous sampling day (ranging from ≃ 67 % to ≃ 87 %). In the liver (Fig. 1.1B and 1.2B), for both the experimental series, Cu content showed almost no differences between treatment and control; however at day seven the metal content increased in similar amounts for all the treatments compared with the controls (ranging from ≃ 60% to ≃ 135 %). In the remaining carcasses (Fig. 1.1C and 1.2C), by the end of the exposure period, a significant increase was observed in the treatments $Cu_{fix}/Cd_{10\text{-}25}$ compared with the control. In the treatment Cd_{fix}/Cu_{50}, Cu increased significantly compared with the control from day three onwards. Moreover, at day seven, the metal content was higher in the treatments Cu_{fix}/Cd_{25} and Cd_{fix}/Cu_{25} compared to day one (Fig. 1.1C and 1.2C).

For both experimental series, a fast Cu accumulation was observed in the gills during the first day in all treatments. From day one onwards, Cu accumulation continued at a slightly lower pace, increasing linearly in time (See supplementary information, SI-Fig 1 A and B). During experimental series $Cu_{fix}/Cd_{var,}$ there seems to be a steady Cu net accumulation rate (≃ 2.6, 1.5 and 1.4 nmol g^{-1} dw h^{-1} for day one, three and seven respectively), which is not affected by Cd levels in the water (SI-Fig 1 A-1 and SI-Table 3). However, looking at experimental series Cd_{fix}/Cu_{var}, the accumulation appears to reach a limiting value at the highest Cu exposure concentration during the first days, which is less pronounced at day seven (SI-Fig 1 A-2 and SI-Table 4). By the end of the experiment, Cu accumulation in both the experimental series in terms of percentage of increase was in the order gills > liver > carcass, whereas in

terms of absolute values the order was liver > gills > carcass. In muscle and brain tissue, no statistically significant accumulation of Cu was observed for both exposures (SI-Table 5 and 6).

3.1.2. Cadmium bioaccumulation

Cadmium concentrations in the gills were nearly always significantly higher in the treatments compared to the control for the exposure series Cu_{fix}/Cd_{var} (Fig. 2.1A). Moreover, Cd accumulation showed a concentration dependent increasing trend linked to the waterborne metal concentrations. Furthermore, Cd in the gills significantly increased in all the treatments compared to the previous sampling day from day three onwards (Fig. 2.1A). Throughout exposure Cd_{fix}/Cu_{var} (Fig. 2.2A), Cd concentrations in the gills were significantly elevated in almost all the treatment groups compared to the control from day one onwards. A significantly lower Cd accumulation was observed with increasing waterborne Cu concentrations. Cadmium concentrations increased significantly in the treatments $Cd_{fix}/Cu_{10\text{-}25}$ at day three compared with day one, while for treatment Cd_{fix}/Cu_{50} this only occurred after seven days (Fig. 2.2A).

For liver and carcass similar, but less pronounced Cd accumulation trends were observed in both exposure series (Fig 2.1B, 2.2B, 2.1C and 2.2C). For the exposure Cu_{fix}/Cd_{var}, significantly elevated Cd levels in the liver (Fig. 2.1B) compared to the control were observed in treatment Cu_{fix}/Cd_{50} from day one onwards and treatment Cu_{fix}/Cd_{25} from day three onwards. A significantly higher Cd accumulation in the liver of fish exposed to higher Cd concentrations was most evident at the end of the exposure. Cd content significantly increased compared to day one from day three onwards for treatment Cu_{fix}/Cd_{50} and at day seven for treatment Cu_{fix}/Cd_{25}. For exposure Cd_{fix}/Cu_{var} (Fig. 2.2B), significantly elevated liver Cd levels compared to the control were observed in treatment Cd_{fix}/Cu_{10} from day three onwards and Cd_{fix}/Cu_{25} after seven days. A significant difference in Cd accumulation among treatments was only observed after seven days. Cadmium levels significantly increased compared to day three in treatment Cd_{fix}/Cu_{10} and Cd_{fix}/Cu_{25} after seven days.

During exposure Cu_{fix}/Cd_{var}, the Cd concentration in the carcass (Fig. 2.1C) of treatment Cu_{fix}/Cd_{25} and Cu_{fix}/Cd_{50} showed a significant increase compared to the control from day three onwards. At the end of the exposure, Cd concentrations in the carcass were significantly elevated in treatment Cu_{fix}/Cd_{25} and Cu_{fix}/Cd_{50} compared to day one (Fig. 2.1C). Regarding exposure Cd_{fix}/Cu_{var}, Cd concentrations in the carcass were significantly elevated compared to the control in treatment Cd_{fix}/Cu_{10} from the first day onwards and from day three onwards in treatment Cd_{fix}/Cu_{25} and Cd_{fix}/Cu_{50} (Fig. 2.2C). Significantly higher Cd accumulation was observed in treatment Cd_{fix}/Cu_{10} compared to Cd_{fix}/Cu_{25} and Cd_{fix}/Cu_{50} from day one onwards (Fig. 2.2C). A significant increase in Cd content for all treatments was observed after three days compared to day one with a further increase after seven days for treatment Cd_{fix}/Cu_{10} (Fig. 2.2C).

Cadmium accumulation increased both through time and among the different exposure levels without reaching steady-state in the Cu_{fix}/Cd_{var} exposures (SI-Fig 2 A-1 and B-1). Although metal concentrations increased with time in fish exposed to Cd_{fix}/Cu_{var} (SI-Fig 2 A-2), a clear dose dependent inhibition of Cu on Cd levels can be observed, with a fast reduction in Cd uptake at the highest Cu exposure level. The accumulation rates for Cd in fish exposed to $Cu_{fix}/Cd_{10\text{-}25\text{-}50}$ were $\simeq$ 0.3, 0.6 and 1/0.8 nmol g^{-1} dw h^{-1}, respectively for day one and three, whereas the accumulation rates dropped to 0.1, 0.3 and 0.4 nmol g^{-1} dw h^{-1}, respectively at the end of the experiment. In fish exposed to the $Cd_{fix}/Cu_{10\text{-}25\text{-}50}$ scenario, the accumulation rates expressed in nmol g^{-1} dw h^{-1}were 1.1, 0.6 and 0.4, respectively for day one and 0.9, 0.6

and 0.2, respectively at day three. A further decrease was observed at day seven were the accumulation rates were 0.5, 0.3 and 0.2 nmol g^{-1} dw h^{-1} respectively.

During the first day of exposure Cd_{fix}/Cu_{var}, Cd concentrations seem to level off into a plateau with almost equal inhibition at 25 % and 50 % LC_{50} for Cu (SI-Fig 2 B-2). However, this effect disappeared from day three onwards with a dose dependent inhibition observed for all Cu concentrations used (SI-Fig 2 B-2).

Similar to the results for Cu, the observed accumulation pattern in both exposures was gills > liver > carcass both in terms of relative and absolute values. In muscle and brain tissue, Cd levels stayed below the detection limit in both exposures (SI-Table 5 and 6).

3.2. Expression of MTs and antioxidant enzymes

A clear increased expression of the gene coding for metallothionein in the gills was observed in all treatments compared to the control from day one onwards in both exposure series (Fig 3.1A and 3.2A). In experimental series Cu_{fix}/Cd_{var}, a significantly higher MT gene expression was observed in treatment Cu_{fix}/Cd_{50} compared to Cu_{fix}/Cd_{25} after three days and Cu_{fix}/Cd_{10} after seven days (Fig. 3.1A). For exposure series Cd_{fix}/Cu_{var} no significant differences in MT gene expression were observed among treatments (Fig. 3.2A). The MT gene expression in the liver showed a statistically significant increase compared to the control after three days for all treatments in the Cu_{fix}/Cd_{var} series (Fig. 3.1B). A significant increase in liver MT gene expression compared to day one was observed for all treatments after three days with a subsequent significant decrease at day seven (Fig. 3.1B). For experimental series Cd_{fix}/Cu_{var} (Fig. 3.2B), a significantly higher transcription of the MT gene in the liver compared to the control was observed for treatment Cd_{fix}/Cu_{25} after three days and Cd_{fix}/Cu_{50} after seven days. Gene expression significantly increased compared to day one for treatment Cd_{fix}/Cu_{25} and Cd_{fix}/Cu_{50} after three days (Fig. 3.2B). After seven days a significant increase compared to day three was observed for treatment Cd_{fix}/Cu_{50} (Fig. 3.2B).

Relative GR mRNA abundance in the gills was almost doubled throughout experimental series Cu_{fix}/Cd_{var} in all treatments compared to the control (Fig. 4.1A). Moreover, no statistically significant differences in relative GR gene expression among treatments was observed. During exposure Cd_{fix}/Cu_{var} (Fig. 4.2A), a significantly increased relative GR mRNA abundance compared to the control was observed on nearly all sampling days. After seven days, relative GR mRNA abundance in treatment Cd_{fix}/Cu_{50} was significantly elevated compared to the control and both other treatments. Moreover, a significant increase compared to day three and one was also observed (Fig. 4.2A).

Regarding the expression of SOD in the gills, a significant increase in relative mRNA abundance compared to the control was observed from day three onwards in treatment Cu_{fix}/Cd_{10} and on day seven for treatment Cu_{fix}/Cd_{25} (Fig. 4.1B). During exposure Cd_{fix}/Cu_{var} a significant increase in relative SOD mRNA was observed after seven days in treatment Cd_{fix}/Cu_{25} and Cd_{fix}/Cu_{50} (Fig. 4.2B). Moreover, for both the exposure scenarios, no significant differences were observed among the treatments during the same sampling day. Furthermore, the gene expression of treatment Cd_{fix}/Cu_{50} at day seven is significantly higher compared with day one.

Concerning the expression of CAT in the liver, no significant differences were observed between the control and the treatment for fish exposed to Cu_{fix}/Cd_{var} (Fig. 4.1C). A significant decrease in relative mRNA abundance compared to the control was observed on day one and three for treatment Cd_{fix}/Cu_{50}; however, by day seven the levels were similar to the control (Fig. 4.2C).

Finally, for GST expression in the liver, a significant decrease in relative mRNA abundance compared to the control was observed for treatment Cu_{fix}/Cd_{10} after three days (Fig. 4.1D).

During exposure Cd_{fix}/Cu_{var} (Fig. 4.2D), a significant increase compared to the control was observed in treatment Cd_{fix}/Cu_{25} after one day and a significant decrease in treatment Cd_{fix}/Cu_{50} after three days. The GST mRNA abundance significantly decreased compared to the first sampling day, in treatment Cd_{fix}/Cu_{10} and Cd_{fix}/Cu_{25} after three days (Fig. 4.2D).

In gills, no statistically significant changes in relative GST and CAT mRNA abundance were observed between treatments and control during both exposure series (SI-Table 8 and 9). In liver, no statistically significant changes in relative SOD and GR mRNA abundance between treatments and control were observed during both exposure series (SI-Table 8 and 9).

3.3. Effects of metal exposure on ionoregulation

3.3.1. Sodium

The Na concentration in the gills showed a similar trend in the two experimental series, with a significant loss in the treatments from day one onwards (Fig. 5.1A and 5.2A). No significant difference in Na loss among treatments was observed. Changes in the electrolyte content for fish exposed to the same treatments started to become evident after day three, compared with the previous sampling day for the exposure Cu_{fix}/Cd_{var} (Fig. 5.1A), whereas for fish exposed to a variable concentration of Cu, the decrease was more accentuated at day seven compared with the previous days (Fig. 5.2A). In the liver a significant Na decrease compared to the control was observed from day one onwards for nearly all the treatments for fish exposed to a fixed amount of Cu (Fig. 5.1B). After seven days, a significantly lower Na concentration compared to day one was observed in treatment Cu_{fix}/Cd_{10} and Cu_{fix}/Cd_{25} (Fig. 5.1B). In the second experimental series, a significant Na loss compared to the control was observed in treatment Cd_{fix}/Cu_{25} and Cd_{fix}/Cu_{50} after seven days (Fig. 5.2B). A significant sodium decrease compared to day one was observed in treatment Cd_{fix}/Cu_{50} at day seven (Fig. 5.2B). In the muscle (Fig. 5.1C and 5.2C) a comparable trend can be observed between the two experimental series, with a significant Na loss in the treatments compared with the control only by the end of the experiment. A significantly lower Na concentration compared to day one was only observed in fish from treatment Cu_{fix}/Cd_{10} and Cu_{fix}/Cd_{50} after seven days (Fig. 5.1C). In the brain a significant Na decrease was observed for all the treatments of exposure Cu_{fix}/Cd_{var} from day three onwards (Fig. 5.1D) which was also significant compared to day one (Fig. 5.1D). For experimental series Cd_{fix}/Cu_{var} a Na loss was observed only for fish exposed to the highest concentration of Cu at day seven (Fig. 5.2D). Finally, for the carcass (Fig. 5.1E and 5.2E), a significant decrease in Na levels was observed for all treatments from day three onwards in exposure series Cu_{fix}/Cd_{var} (Fig. 5.1E) and from day one onwards in exposure series Cd_{fix}/Cu_{var} (Fig. 5.2E).

3.3.2. Potassium

Potassium concentrations in the liver during experimental series Cu_{fix}/Cd_{var} significantly decreased for all treatments compared to the control from day three onwards (Fig. 6.1B). A significantly lower liver K concentration compared to day one was observed after three days in treatment Cu_{fix}/Cd_{10} and Cu_{fix}/Cd_{50} and after seven days in treatment Cu_{fix}/Cd_{25}. Furthermore, after seven days a further K decrease compared to day three was observed in treatment Cu_{fix}/Cd_{10}. During experiment Cd_{fix}/Cu_{var} (Fig. 6.2B), liver K concentrations were significantly lower in treatment Cd_{fix}/Cu_{25} and Cd_{fix}/Cu_{50} compared to the control from day three onwards. For treatment Cd_{fix}/Cu_{10} this decrease became significant at the end of the experiment. Potassium content in the liver was significantly lower compared to day one in treatment Cd_{fix}/Cu_{10} after three days and treatment Cd_{fix}/Cu_{50} after seven days (Fig. 6.2B).

In the brain, K content significantly decreased compared to the control group for all treatments from day one onwards during exposure Cu_{fix}/Cd_{var} (Fig. 6.1D). A significant decrease in K concentrations compared to day one was observed in treatment Cu_{fix}/Cd_{10} and Cu_{fix}/Cd_{25} after seven days (Fig. 6.1D). For exposure Cd_{fix}/Cu_{var} (Fig. 6.2D), K concentrations significantly decreased compared to the control in all treatments after seven days. Moreover, for treatment Cd_{fix}/Cu_{50} the decrease was already significant after one day (Fig. 6.2D). A significantly lower brain K concentration compared to day one was observed in treatment Cd_{fix}/Cu_{50} after three days and Cd_{fix}/Cu_{25} after seven days (6.2D).
For the remaining carcasses a significant K loss was only observed for all treatments compared to the control group at the end of exposure Cu_{fix}/Cd_{var} (Fig. 6.1E), while in the Cd_{fix}/Cu_{var} (Fig. 6.2E), a significant loss compared to the control was observed for treatment Cd_{fix}/Cu_{50} only after seven days.
Gills and muscle samples did not show any significant differences in K content compared to the control throughout both experiments (Fig. 6.1A, 6.2A, 6.1C and 6.2C).

3.3.3. Calcium and Magnesium

Calcium concentration in gills significantly decreased compared to the control after seven days in treatment Cu_{fix}/Cd_{50} (Fig. 7.A). For the liver, a significant decrease compared to the control was observed in treatment Cu_{fix}/Cd_{50} after three days, but not after seven days (Fig. 7.C).
Regarding the Mg content in the gills, no significant changes were observed during exposure Cu_{fix}/Cd_{var} (Fig. 7.B). For the liver, a significant decrease for all treatment groups compared to the control was evident after seven days (Fig. 7.D). Moreover, the Mg content declined significantly compared to day one, in treatment Cu_{fix}/Cd_{10} and Cu_{fix}/Cd_{50} at day three and treatment Cu_{fix}/Cd_{25} at day seven (Fig. 7.D). A further liver Mg concentration decrease compared to day three was observed in treatment Cu_{fix}/Cd_{10} after seven days (Fig. 7.D). For all other tissues, no significant changes in the Ca or Mg content were observed during both experimental series (SI-Table 5 and 6).

3.3.4. Gene expression of ion channels in the gills

The expression of the CTR1 gene in the gills increased compared to the control in all treatments at day one and day seven during exposure Cu_{fix}/Cd_{var} (Fig. 8.1A). After three days, the expression significantly decreased compared to day one in treatment Cu_{fix}/Cd_{10} and Cu_{fix}/Cd_{25} (Fig. 8.1A). During exposure Cd_{fix}/Cu_{var} the CTR1 gene expression significantly increased in all treatments compared to the control for all sampling days, except for treatment Cd_{fix}/Cu_{50} on day one and treatment Cd_{fix}/Cu_{10} on day three (Fig. 8.2A). After seven days, a significantly higher CTR1 gene expression was observed in treatment Cd_{fix}/Cu_{25} and Cd_{fix}/Cu_{50} compared to treatment Cd_{fix}/Cu_{10} (Fig. 8.2A). Furthermore, after seven days, the expression significantly increased compared to day three in treatment Cd_{fix}/Cu_{25} and treatment Cd_{fix}/Cu_{50} (Fig. 8.2A).

Regarding H^+-ATPase gene expression, a significant increase in the transcription was observed in all treatments at day one and seven during exposure Cu_{fix}/Cd_{var} (Fig. 8.1B). During exposure Cd_{fix}/Cu_{var}, a significant increase was observed for all treatments compared to the control after seven days (Fig. 8.2B). Moreover, for treatments Cd_{fix}/Cu_{50} this increase was already significant from day one onwards and for treatment Cd_{fux}/Cu_{25} it was also significant on day one (Fig. 8.2B). Considering Na^+/K^+-ATPase gene expression, a significant increase in mRNA abundance compared to the control was observed in treatment Cu_{fix}/Cd_{10} after one day (Fig. 8.1C). After three days, the relative Na^+/K^+-ATPase mRNA abundance significantly decreased in all treatments compared to day one (Fig. 8.1C). During exposure Cd_{fix}/Cu_{var}, a significant increase in relative Na^+/K^+-ATPase mRNA abundance compared to the control was

observed after one day in all treatments (Fig. 8.2C). After three days, relative Na^+/K^+-ATPase mRNA abundance significantly decreased in treatment Cd_{fix}/Cu_{10} and Cd_{fix}/Cu_{25} compared to day one (Fig. 8.2C).

Regarding NHE-2, a significant decrease in relative mRNA abundance compared to the control was observed from day three onwards for all treatments during exposure Cu_{fix}/Cd_{var} (Fig. 8.1D). During exposure Cd_{fix}/Cu_{var}, a significant decrease of the relative NHE-2 mRNA abundance compared to the control was observed on day three for treatment Cd_{fix}/Cu_{25} and from day three onwards in treatment Cd_{fix}/Cu_{50} (Fig. 8.2D).

4. Discussion

We hypothesized that metal bioaccumulation and induction of protective mechanisms would occur. Results show that defensive mechanisms in common carp were able to respond adequately to minimize adverse effects and mortality. As expected throughout the experiment mortality was limited to treatment Cd_{fix}/Cu_{50} and only three fish ($\simeq$ 8% of the population of one experimental series) died. The relatively low mortality could be explained by the short exposure period, the relatively tolerable Na loss and the activation of defensive mechanisms.

4.1. Dynamics of Cu and Cd bioaccumulation

4.1.1. Copper bioaccumulation

Not surprisingly, our data confirmed our initial hypothesis that metals would accumulate faster in gills and liver compared to other tissues. As expected, in fish exposed to a fixed amount of Cu, the content of this metal in the gill tissue, increased in comparable amounts for all the treatments ($Cu_{fix}/Cd_{10\text{-}25\text{-}50}$) at each sampling day, showing the importance of the exposure time on metal accumulation. After one week of exposure, for the Cu_{fix}/Cd_{var} series, the variable amount of Cd in the water showed relatively little effect on gill Cu accumulation (net Cu content by the end of the experiment was approximately 230, 240 and 213 nmol/g dw for treatment $Cu_{fix}/Cd_{10\text{-}25\text{-}50}$ respectively). For the experimental series Cd_{fix}/Cu_{var} a more marked Cu net accumulation, proportional to the metal exposure concentration in the water, was expected by the end of the experiment. Probably this discrepancy was due to the presence of Cd, which seemed to stimulate Cu accumulation at the lower Cu exposure concentrations.

When comparing the results obtained in the single exposure scenario using comparable metal concentrations, in which the net accumulated values after one week were approximately 112, 182 and 308 nmol/g dw for Cu, and 81, 137 and 267 nmol/g dw for Cd (Castaldo et al. 2020, Castaldo et al., under review) with results obtained in the binary mixture, it can be noticed that Cu content is slightly higher in the treatments $Cd_{fix}/Cu_{10\text{-}25}$ (respectively $\simeq$ 44-32 %) but that is not for the case of exposure to Cd_{fix}/Cu_{50}. Also in the Cu_{fix}/Cd_{var} exposure series, Cu accumulated to values slightly higher than those found for 25% of the 96 h-LC_{50} in the single Cu exposures ($\simeq$ 17-32%). This confirms that there was no systematic inhibiting effect of Cd on Cu accumulation, if anything, it was slightly stimulating (except at the highest Cu exposure). Results on Cu accumulation inhibition/stimulation by Cd are often inconsistent. A reduction of Cu uptake in presence of Cd was demonstrated in water flea (*Daphnia magna*) and in rainbow trout (Komjarova and Blust 2008, Niyogi et al. 2015). However, in another study, in which zebrafish were exposed to an increasing concentration of Cd (0.01, 0.05, 0.2 µM) plus a fixed concentration of Cu (0.02 µM), the presence of Cd did not alter Cu uptake (Komjarova and Blust 2009). Moreover, in rainbow trout a stimulation of Cu uptake in presence of Cd occurred (Brix et al. 2016). Therefore, the explanation of shared transporters and non-specific competition for binding sites seems reasonable (Niyogi et al. 2015). In fact, even though Cd and Cu are considered to be respectively the Ca^{2+} and Na^+ antagonists (Grosell et al. 2002, Niyogi and Wood 2004), several studies provided evidence of shared uptake routes of these metals in fish gills via the ECaC, DMT1 and Zip-8 (Cooper et al. 2007, Alsop and Wood 2011, Komjarova and Bury 2014, Niyogi et al. 2015). Alternatively, effects might go unnoticed, since

after the first day with faster accumulation rates, the Cu accumulation rate seems to attain a steady-state value which is similar over all exposure conditions.

In gills, the fast Cu accumulation during the onset of the exposure was expected, considering that gills are in direct contact with the water, and the exposure medium is non-complexing (see SI-Table 1 and 2). Similarly, a previous study on rainbow trout and European eel (*Anguilla anguilla*), reported a rapid accumulation of Cu in the gills already after a few hours of exposure (Grosell et al. 1998, Kamunde et al. 2002). Moreover, such a fast accumulation is consistent with the high conditional equilibrium constant for metal ion binding sites on the gill surface (log K_{cond} = 7.4 - 7.8 (dm^3 mol^{-1}), calculated at pH 6.2 and 7.9 with ionic strength of ≃ 1.e-04 and 3.20e-03) (Playle et al. 1993, Brix et al. 2016) and the capacity thereof. However, we have to consider that gills are only a temporary target organ for metal toxicity, as metal ions are subsequently transferred to the liver and kidney for the excretion via the hepatobiliary system (Grosell 2011, Kondera et al. 2014). In fish exposed to Cu_{fix}/Cd_{var} and Cd_{fix}/Cu_{var}, a significant Cu accumulation in the liver was only observed at day seven. During experimental series Cd_{fix}/Cu_{var}, this accumulation appeared to be directly proportional to the external Cu concentration, even though differences among treatments were not statistically significant. Moreover, the pronounced increase in Cu content in both liver and gills after seven days could be related to the hepatobiliary excretion no longer being able to compensate for the increased metal bioaccumulation through the gills. Regarding Cu accumulation in the carcass, the transient increase reported for both the exposure scenarios seems to follow the pattern observed for liver with a slight delay in the accumulation, supporting the hypothesis that regulatory mechanism are struggling to keep up with the continuous Cu uptake via the gills. No significant accumulation of Cu in muscle tissue was observed in the present study. A lack of Cu accumulation in the muscle of common carp was also observed by De Boeck et al. (1997), suggesting that the metal accumulation in the muscle becomes significant when the storage capacity of the liver is exceeded (Laurén and McDonald 1987). In the brain no Cu accumulation was reported, this is in accordance with what observed by Shaw et al. (2012) and with the thought that Cu accumulation from metal salts in fish brain is slow (Handy 2003, Shaw et al. 2012).

Several mechanisms in vertebrates are known to play a role in Cu homeostasis, such as the CTR1 and the Cu-ATPase (Anni et al. 2019). The CTR1 has been proposed as a Cu^+ transporter which is insensitive to external Na^+ concentrations (Mackenzie et al. 2004, Craig et al. 2010, Komjarova and Bury 2014). According to Grosell and Wood (2002) the copper uptake pathway which is sensitive to external Na^+ concentration dominates in environments with a Na^+ deficiency, whereas the Na^+ insensitive copper uptake pathway dominates when Na^+ concentrations are above 200 µmol l^{-1} (Grosell and Wood 2002). The regulation of this transporter is unclear and results are often contradictory (Boyle et al. 2011). The transcript level of CTR1 is downregulated in the intestine of sea bream in response to a high copper diet, whereas that is not the case during waterborne Cu exposure (concentration representing the 25 % 96 h LC_{50}) (Minghetti et al. 2008). Similar to what was found by Komjarova and Bury (2014) in zebrafish exposed to Cu, we observed an increased CTR1 gene expression in common carp. In Cu_{fix}/Cd_{var} series a significant increase in CTR1 mRNA abundance occurred at day one and seven, whereas in the Cd_{fix}/Cu_{var} series the gene expression was almost continuously increased in all treatments when compared to the control. The observed increment, especially at day seven, seems to be dependent on exposure media Cu levels. Also in zebrafish gills exposed to 0.016 µM of Cu, an increase in CTR1 gene expression was reported (Leung et al. 2014). Similarly, in yeast the transcript of CTR-type transporters are regulated by Cu levels (Labbé et al. 1997), whereas this is not observed in mammals (Lee et al. 2000). According to our results, in addition to the influence of the Cu concentrations, one can assume that the observed increased expression could be related to changes in internal electrolyte concentrations. Moreover, in fasted fish, an increase in cortisol levels can occur (Vijayan and Moon 1992, Hashemi et al. 2008), playing a role in up-regulating CTR1 mRNA expression as suggested by Tellis et al. (2012). However, a downregulation of the Cu

transporter gene would be expected to slow down metal accumulation and prevent potential toxic effects.

4.1.2. Cadmium bioaccumulation

Unlike Cu, Cd is a xenobiotic, which does not fulfil any known metabolic role and is considered as a non-essential metal (Matsuo et al. 2005). In the gills a time and dose dependent increase can be observed. Furthermore, an antagonistic-like interaction between accumulation of the two metal ions was obvious, since higher water Cu concentrations resulted in lower levels of Cd accumulation (Cd content by the end of the experiment was approximately 79, 50 and 29 nmol/g dw for treatment $Cd_{fix}/Cu_{10\text{-}25\text{-}50}$ respectively).

Regarding Cd in presence of Cu, the accumulation was up to 4 times lower compared to the single exposure scenario. These results are also reflected in the decreasing accumulation rates for Cd with increasing Cu exposure concentrations. A similar inhibition compared to the single exposures was also observed in an earlier study, in which common carp were exposed to a ternary mixture of 10% of the 96 h-LC_{50} of Cu, Zn and Cd (Castaldo et al. 2020). Moreover, an antagonistic inhibition of Cd uptake in the presence of Cu was reported in several other fish species such as Nile tilapia (*Oreochromis niloticus*), rainbow trout and zebrafish exposed to a Cd/Cu mixture (Eroglu et al. 2005, Komjarova and Blust 2009, Brix et al. 2017). The presence of shared uptake routes for Cd^{2+} and Cu^{2+}, and non-specific competition for binding sites in fish gills likely explain the antagonistic like effect of Cu on Cd uptake (Cooper et al. 2007, Alsop and Wood 2011, Komjarova and Bury 2014). In general, Cd accumulated fast in the gills (Vinodhini and Narayanan 2008) and considering the very low background levels, accumulation was significant from the beginning, with the exception of fish exposed to Cd_{fix}/Cu_{50}.

In contrast to Cd accumulation in the gills, accumulation in the liver was more differentiated across treatments: Cd increased in the liver from day one in the treatment Cu_{fix}/Cd_{50}, followed by the Cu_{fix}/Cd_{25} treatment from day three onwards. Similar to a previous study with common carp exposed to a ternary mixture (Castaldo et al. 2020), no differences were observed in fish exposed to the lowest Cd concentration. Probably, this is due to efficient excretion processes (faeces, mucosal sloughing and hepatobiliary excretion) (McGeer et al. 2011). In contrast to exposure Cu_{fix}/Cd_{var}, fish exposed to Cd_{fix}/Cu_{var} mainly showed a significant Cd accumulation in the liver at day seven. However, Cd accumulation in the liver occurred only for the treatments $Cd_{fix}/Cu_{10\text{-}25}$ reflecting the accumulation pattern of the gills. Regarding the Cd accumulation in the carcasses, a similar pattern compared to gills and liver was reported for both experimental series. The metal concentration seems to follow a transient increase starting at day three for almost all the treatments. However, we have to take into account that no Cd accumulation was reported in the muscle and in the brain. Therefore, one can hypothesize that this limited Cd accumulation could be explained by metal adsorption to the skin.

The rapid and substantial Cd accumulation in the gills reflects their role as the primary uptake site of metal ions during waterborne exposures, and indicates the vulnerability of these tissues, (Benhamed et al. 2016). The rapid Cd accumulation, similar to Cu, is consistent with the very high affinity that Cd has for gill binding sites (conditional log *K* of 8.6 (dm^3 mol^{-1}), calculated at pH 6.2 and ionic strength of 1.e-04) (Playle et al. 1993, Playle 2004) and the capacity thereof, together with the non-complexing nature of our exposure media. Considering the binding constants, one can assume that relatively more Cd, rather than Cu, should bind to the gill surface. However, our results showed a higher Cu-compared to Cd accumulation. Therefore, we can hypothesize that both metals entered the cell, but Cu displaced Cd from MTs due to a higher affinity for the protein (Vašák 1991). Thus, Cd will be subsequently flushed away into the kidney for excretion processes, whereas Cu will remain into the tissue bound to MTs. Moreover, we can also consider firstly, that an antagonistic-like effect, of Cu on Cd uptake due to the shared branchial uptake routes will occur (Alsop and Wood 2011, Komjarova and Bury 2014), secondly that at the same equitoxic concentrations, Cd levels were almost three times

lower compared to Cu, and thirdly that our fish were fasted. In fact a higher Cu accumulation was reported in fish exposed to a reduced food ratio (Hashemi et al. 2008). Therefore, we hypothesize that due to the absence of food, fish tried to compensate for the electrolyte losses, by enhancing the uptake of essential elements from the water, thereby promoting Cu uptake. Moreover, it has been suggested that Cu binding capacity (B_{max}) can vary reflecting changing requirements of this essential metal in growing juvenile fish (Brix et al. 2016).

No significant accumulation of Cd in muscle tissue was observed in the present study. This is in accordance with previous studies that reported a significant Cd accumulation only after several months of exposure (Cinier et al. 1999, Benhamed et al. 2016). Similar to the muscle, no Cd accumulation was observed in the brain. This was unexpected since the potential of Cd to accumulate in the brain of freshwater fish, such as silver catfish and zebrafish was pointed out in previous studies (Pretto et al. 2010, Al-sawafi et al. 2017).The lack of both Cu and Cd accumulation in these tissues indicates on the one hand that storage organs such as the liver were not saturated and on the other hand the ability of common carp to handle metal excesses. However, a longer exposure is needed to validate this thought.

4.2. Defensive mechanisms

Metallothioneins (MTs) are cysteine rich proteins which play an important role in metal ion homeostasis: their binding affinity for metal ions can reduce intracellular free metal ion concentrations thereby providing a protective role (Hamilton and Mehrle 1986, De Boeck et al. 2003). The metal binding strength of metallothioneins follows the order $Hg^{2+} > Cu^{+} > Cd^{2+} > Pb^{2+} > Zn^{2+} > Co^{2+}$ (Vašák 1991). In the present study, all the exposure conditions showed MT gene induction. In general, the MT mRNA expression was always increased in the gills, whereas that was not the case in the liver. In fact, the MT gene expression was delayed in the liver until day three. The increase in MT gene expression occurred concurrently with a significant metal accumulation for both the tissues. Several studies have pointed out the important role of MTs as metal scavengers and the relationship between metal accumulation and MT levels in different tissues (De Smet et al. 2001, De Boeck et al. 2003). The fast accumulation of Cu and Cd in the gills may have triggered the induction of the MT gene. Considering that background MT levels differ between the different tissues, with lower values in the gills compared to the liver (Hashemi et al. 2008), we can assume that the rapid increase in gene expression in the gills was a response of the fish to induce the synthesis of MTs in order to increase the protein levels. This fast response in common carp, as suggested by De Boeck et al. (2003), is clearly an advantage considering that extensive damage is usually caused by Cu toxicity during the first hours and days of exposure (McDonald and Wood 1993). Regarding the liver, the MT gene expression peak, observed at day three, followed by a decrease at day seven, could suggest that a temporary elevated protein synthesis was sufficient to cope with the metals, at least during this one week exposure. Common carp is known to quickly adapt to prolonged metal ion exposures, only increasing defensive mechanisms when needed (Martinez et al. 2004, Pillet et al. 2019).

As already mentioned, in addition to MTs, various antioxidant enzymes are present in cells to cope with deleterious effects caused by ROS (Wang et al. 2010). In the present work, we investigated the relative gene expression of SOD, CAT, GR and GST both in the liver and in the gills. The results obtained in the gills for both the exposure series are quite similar. Glutathione reductase is an enzyme involved in the renewal of GSH, and together with GST, it plays a key role in the GSSG/GSH balance (Dautremepuits et al. 2009). We observed an upregulation of the GR gene, similar to what was reported for sea bream exposed to Cu (Minghetti et al. 2008) and obscure pufferfish (*Takifugu obscurus*) exposed to Cd (Kim et al. 2010). Moreover, Kim et al. (2010), proposed that GR is one of the main antioxidants against Cd-induced oxidative stress in *obscure pufferfish*, since its transcript had the highest expression level in all examined tissues. It is known that common carp rely on GSH, which can bind metal ions, as a first line of defence (Eyckmans et al. 2011). Therefore, an increase in the GR gene expression is expected to reduce the level of oxidized glutathione. The obtained results, similar to what observed in a previous study (Castaldo et al. 2020),

suggested that a saturation of base levels of MT and GSH occurred, due to their ability to bind metal ions (Lange et al. 2002), and that a continuous production of the proteins is needed to handle the increasing amount of metals in the gills.

Regarding the SOD, metal ions such as Cd, Cu and Zn have been demonstrated to increase the SOD gene transcript (Sanchez et al. 2005, Cho et al. 2006). In our study, a general increase of the SOD gene occurred in all treatments compared to the control after seven days of exposure. Superoxide dismutase, together with CAT are considered as the first line of defence against ROS (Atli and Canli 2010, Weydert and Cullen 2010, Pillet et al. 2019). Therefore, we can assume that this transcription increase, which occurred concurrently with a drastic metal accumulation, is an attempt by the fish to boost its internal defences.

In the liver, no differences were observed in the expression of the GR and SOD genes. Considering that metal levels in the liver only started to increase after three or seven days, this could be an indication that metal levels remained below the threshold required to significantly increase ROS production. Regarding the CAT mRNA abundance, a decrease was observed during the first three days, followed by a subsequent recovery. This shows, on the one hand the susceptibility of common carp to elevated Cu and Cd concentrations and on the other hand the ability to quickly adapt to a stressful situation. In other studies, increases as well as decreases in CAT activity have been reported (Jia et al. 2011, Díaz-de-Alba et al. 2017, Pan et al. 2018). However, our trend is in accordance with Pillet et al. (2019) and indicates that common carp are able to adapt rapidly to metal ion exposure.

From the obtained results, we can conclude that gills, as expected, are the most vulnerable tissue. In fact the gills were the tissue with the highest percentage of metal accumulation, therefore a higher demand of defensive proteins is needed to counteract the high metal accumulation. Moreover, the gene expression responses of defensive mechanisms suggest fast adaptability of common carp towards oxidative stress.

4.3. Changes in electrolyte levels and organism responses

Metal ions are known to interfere with electrolyte ion homeostasis, due to competition at the uptake sites (Čelechovská et al. 2007, Niyogi et al. 2015). Among the electrolytes, Na^+ is the major cation of the extracellular fluid (Sathya et al. 2012). In agreement with our initial hypothesis, decreased levels of Na were observed within the first day of exposure for both the experimental series. The net Na loss observed in the gill by the end of the experiment, in fish exposed to Cu_{fix}/Cd_{10} and Cd_{fix}/Cu_{10} was respectively around 159 and 117 µmol/g dw. This Na loss was slightly higher than in fish exposed to a ternary mixture of 10% 96 h LC_{50} Cu, Zn and Cd, in which the Na loss was 78 µmol/g dw (Castaldo et al. 2020). Considering that Cd alone did not alter Na^+ influx in rainbow trout (Birceanu et al. 2008), nor Na levels in common carp gills (Delahaut et al. 2019), this electrolyte decrease cannot be explained by additive effects. It is known that both Cu and Cd can inhibit the Na^+/K^+-ATPase activity through the binding at the Mg^{2+} binding sites and the -SH groups, respectively (Lionetto et al. 2000, Grosell et al. 2002, Handy et al. 2002). Therefore, it is likely that the inhibition of Na^+/K^+-ATPase activity caused by both metal ions, gradually lead to an increased Na^+ content in the gill intracellular fluid (ICF), resulting in a reduced water-gill ICF electrochemical gradient for Na^+ entry via apical channels (Birceanu et al. 2008).

As reported in previous studies, and in different species such as Nile tilapia and rainbow trout, gills are the most affected tissue in waterborne exposures (Grosell and Wood 2002, Mackenzie et al. 2004, Atli and Canli 2011, Niyogi et al. 2015). A drastic loss in total Na can result in death, however different species can tolerate different percentages of loss. For example the threshold for rainbow trout and yellow perch is set to 30 % and 40 % loss of whole body electrolyte content, respectively (Taylor et al. 2003), whereas gibel carp can tolerate losing up to the 45% of their whole body total Na content (De Boeck et al. 2010). In the present study the gill loss of total Na at day seven ranged between 33 and 45 %, whereas in carcass the Na decrease ranged between 24 and 36 %. However, Na levels in the gill of control animals

slightly increased from day one to seven (approximately 15%) which explains the high percentage (45 %) of Na loss encountered in fish gills exposed to Cu_{fix}/Cd_{var}.

Sodium can enter the gills through a putative Na^+-channel powered by H^+-ATPase, a Na^+/Cl^- cotransporter and the NHEs (McCormick 2001, Kumai and Perry 2012). In order to understand mechanisms that act to maintain Na^+-homeostasis, we analysed the expression of genes coding for Na^+/K^+-ATPase, H^+-ATPase and the NHE-2 in the gills. An upregulation of the Na^+/K^+-ATPase gene expression was recorded in fish exposed to Cd_{fix}/Cu_{var} after one day for all the treatments compared to the control. In agreement with Boyle et al. (2011), we hypothesize that the increase in the Na^+/K^+-ATPase gene expression could be a compensatory mechanism to counteract the inhibition of the enzyme activity, caused by metals as already reported by several authors (De Boeck et al. 2001, Eyckmans et al. 2011) for the inhibition of the enzyme activity.
The active uptake of Na^+ in freshwater fish is necessary for ionic homeostasis, and as already mentioned, Na^+ can be taken up via a Na^+/H^+ exchanger or via a putative Na^+-channel coupled with H^+-ATPase (Wilson et al. 2000). Several NHEs have been identified and NHE-2 has been proposed as a candidate for the Na^+-sensitive component of Cu^+ uptake (Mackenzie et al. 2004, Craig et al. 2010, Komjarova and Bury 2014). In contrast with the observations reported by Komjarova and Bury (2014), our results showed a decreasing trend in NHE-2 mRNA abundance in both the exposure series. If NHE-2 can be proposed as a Cu uptake component, this reduction can be interpreted as an attempt by the fish to reduce the direct Cu uptake, and/or as a way to cut down the extrusion of protons in order to minimize the influx of Cu. Nevertheless, the latter assumption is unlikely as the H^+-ATPase expression was upregulated. Alternatively, as described by Grosell (2011), it can be an indirect effect caused by the inhibition of the carbonic anhydrase, which leads to a depletion of the necessary substrate for the exchanger. Theoretically, the lower amount of substrate could lead to a reduced efficiency of the Na^+/H^+- exchanger but not of the H^+-ATPase which is an active transporter. However, in both cases one of the outcomes is a drop of internal Na^+ content, which the organisms try to compensate for by increasing the expression of the gene coding for H^+-ATPase. Nonetheless, the Na^+ loss in the gills has repercussions on the whole-body Na levels.

In contrast to Na^+, K^+ is the major cation of the intracellular environment (Sathya et al. 2012). Potassium homeostasis has been investigated in different species with contradictory results. A whole body K^+ reduction was reported in zebrafish larvae exposed to Cu (1.57 µM) and Cd (10.7 µM) (Alsop and Wood 2011). In contrast, higher levels of K were reported in major carp (*Catla catla*) and *Prochilodus scrofa* exposed to Cu and Cd (Cerqueira and Fernandes 2002, Hassan et al. 2018). Potassium is an important component of the Na^+/K^+-ATPase system, which is involved in maintaining the transepithelial membrane potential. It allows active transepithelial transport and regulates the cell volume (Skou and Esmann 1992). Moreover, the ion exchange mediated by Na^+/K^+-ATPase is crucial to prevent cell swelling (Lodish et al. 2000). The loss of total K, observed in liver, brain and carcass could be related to the ability of metal ions to inhibit the ion-transporting enzymes, leading to cell damage (Suresh et al. 1995, McGeer et al. 2000, Matsuo et al. 2005).

According to previous results obtained in our lab with common carp (Delahaut et al. 2019) we did not expect a loss in total Ca. Nonetheless, after one week of exposure in fish exposed to Cu_{fix}/Cd_{50} an electrolyte decrease occurred in the gills, suggesting a synergistic-like effect between metal ions on Ca loss. For the liver, a significant Ca decrease was observed for treatment Cu_{fix}/Cd_{50} after three days, but not after seven days. This trend is likely due to the considerable variation in the Ca concentration of the liver in fish from the control group throughout the exposure. A competition between Cd^{2+} and Ca^{2+} at the apical and basolateral Ca^{2+}-channel has been reported resulting in hypocalcaemia (McGeer et al. 2000, Niyogi et al. 2015). The significant decrease observed only after one week indicates that both time and the exposure concentrations play a crucial role. Moreover, one can assume that the presence of Cu contributed to this loss. Considering that previous studies on zebrafish showed that Cu

decreases Ca^{2+} uptake and vice versa (Craig et al. 2010, Alsop and Wood 2011), we hypothesize that a competition between Ca^{2+} and Cu^{2+} at the uptake site occurred (Grosell 2011). Moreover, it has been suggested that Cu^{2+} epithelial transport may be via a Ca^{2+} pathway (Alsop and Wood 2011, Komjarova and Bury 2014).

Magnesium, together with Ca^{2+}, is another important cation found in bony tissue and is involved in the activation of numerous enzymes (Bijvelds et al. 1996). A decreased liver Mg content was reported in all the treatments of fish exposed to Cu_{fix}/Cd_{50} after seven days. Since Mg^{2+} is needed for energy metabolism and protein synthesis, a deficiency could occur after prolonged metal exposure due to the higher energy demands or due to competition with metal ions for the protein binding sites (Bijvelds et al. 1996, Matović et al. 2010, Pillet et al. 2019).

5. Conclusions

The main aim of the present study was to investigate the effects of a binomial waterborne metal mixture on bioaccumulation, defensive mechanisms, ion-homeostasis and survival in common carp. One of our initial hypothesis that was confirmed by our data was a quick metal bioaccumulation. Moreover, a dose dependent, non-mutual, antagonistic-like interaction between Cu and Cd uptake was observed. On the one hand Cu showed a marked inhibitory effect on Cd uptake, whereas on the other hand Cd showed a relatively small effect on Cu uptake. Another hypothesis tested in our experiment was the activation of defensive mechanisms, which were activated to a different extent in both gills and liver to protect the fish. In gills genes involved in defensive mechanisms against metal ion toxicity, such as MT and GR, were continuously upregulated compared to the control in order to mitigate possible deleterious effects. In the liver only transient increases in defence mechanisms were observed. As gills are continuously in contact with water, they are more vulnerable to the metal exposure compared to the liver, thus a non-stop production of defences is necessary. Regarding our initial hypothesis on electrolytes levels, a Na loss was confirmed in this study. The loss through the gills affected the whole body Na content, and an impairment of Na homeostasis affected K levels in several tissues as well. Nonetheless, the fish tried to cope with this situation by increasing the expression of the H^+-ATPase and Na^+/K^+-ATPase genes, which are involved in Na^+ homeostasis. In contrast with our expectations, a Ca decrease was reported in the gills, suggesting a synergic-like effect between the two metals on ion-homeostasis. A final hypothesis was that metal mixtures would remain sub-lethal to common carp which was confirmed since only few fish died in the different exposure scenarios. This low mortality rate can be linked to activation of the defence mechanisms present in the fish. In addition, the relatively limited Na loss and the short exposure period could have played a role in fish survival. In conclusion, we can affirm that the negligible mortality, together with the fish responses to a stressful situation, indicate the ability of common carp to cope with these levels of metal pollution, at least for one week. Further studies, in a longer exposure scenario, could provide new insights for unveiling the long-term biological effects of metal pollution on common carp.

Acknowledgments

We are grateful to Steven Joosen for his help with metal and electrolytes analysis, and to the reviewers for their constructive remarks.

Funding

This project was funded by a TOP BOF project granted by the University of Antwerp Research Council (Project ID : 32252) to LB, RB and GDB which included a PhD grant to GC.

Declarations of interest: none

6. Bibliography

Al-sawafi, A., L. Wang, and Y. Yan. 2017. Cadmium accumulation and its histological effect on brain and skeletal muscle of zebrafish. Journal of Heavy Metal Toxicity and Diseases **2**:2.

Alsop, D., and C. M. Wood. 2011. Metal uptake and acute toxicity in zebrafish: common mechanisms across multiple metals. Aquatic Toxicology **105**:385-393.

Anni, I. S. A., Y. D. Zebral, S. B. Afonso, M. B. Jorge, S. I. M. Abril, and A. Bianchini. 2019. Life-time exposure to waterborne copper II: Patterns of tissue accumulation and gene expression of the metal-transport proteins ctr1 and atp7b in the killifish Poecilia vivipara. Chemosphere **223**:257-262.

Atli, G., and M. Canli. 2008. Responses of metallothionein and reduced glutathione in a freshwater fish Oreochromis niloticus following metal exposures. Environmental toxicology and pharmacology **25**:33-38.

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

Atli, G., and M. Canli. 2011. Alterations in ion levels of freshwater fish Oreochromis niloticus following acute and chronic exposures to five heavy metals. Turkish Journal of Zoology **35**:725-736.

Belgisch Staatsblad (Belgian Official Journal). 2015. pages 71554-71588.

Benali, I., Z. Boutiba, D. Grandjean, L. F. De Alencastro, O. Rouane-Hacene, and N. Chèvre. 2017. Spatial distribution and biological effects of trace metals (Cu, Zn, Pb, Cd) and organic micropollutants (PCBs, PAHs) in mussels Mytilus galloprovincialis along the Algerian west coast. Marine pollution bulletin **115**:539-550.

Benhamed, S., F. A. Guardiola, S. Martínez, M. Martínez-Sánchez, C. Pérez-Sirvent, M. Mars, and M. A. Esteban. 2016. Exposure of the gilthead seabream (Sparus aurata) to sediments contaminated with heavy metals down-regulates the gene expression of stress biomarkers. Toxicology reports **3**:364-372.

Bervoets, L., and R. Blust. 2003. Metal concentrations in water, sediment and gudgeon (Gobio gobio) from a pollution gradient: relationship with fish condition factor. Environmental Pollution **126**:9-19.

Bijvelds, M. J., G. Flik, Z. I. Kolar, and S. E. W. Bonga. 1996. Uptake, distribution and excretion of magnesium inOreochromis mossambicus: dependence on magnesium in diet and water. Fish physiology and biochemistry **15**:287-298.

Birceanu, O., M. J. Chowdhury, P. L. Gillis, J. C. McGeer, C. M. Wood, and M. P. Wilkie. 2008. Modes of metal toxicity and impaired branchial ionoregulation in rainbow trout exposed to mixtures of Pb and Cd in soft water. Aquatic Toxicology **89**:222-231.

Blust, R., A. Van der Linden, E. Verheyen, and W. Decleir. 1988. Evaluation of microwave heating digestion and graphite furnace atomic absorption spectrometry with continuum source background correction for the determination of iron, copper and cadmium in brine shrimp. Journal of Analytical Atomic Spectrometry **3**:387-393.

Bopp, S. K., H. K. Abicht, and K. Knauer. 2008. Copper-induced oxidative stress in rainbow trout gill cells. Aquatic Toxicology **86**:197-204.

Boyle, D., C. Hogstrand, and N. R. Bury. 2011. Physiological response to a metal-contaminated invertebrate diet in zebrafish: Importance of metal speciation and regulation of metal transport pathways. Aquatic Toxicology **105**:21-28.

Brix, K. V., M. S. Tellis, A. Crémazy, and C. M. Wood. 2016. Characterization of the effects of binary metal mixtures on short-term uptake of Ag, Cu, and Ni by rainbow trout (Oncorhynchus mykiss). Aquatic Toxicology **180**:236-246.

Brix, K. V., M. S. Tellis, A. Crémazy, and C. M. Wood. 2017. Characterization of the effects of binary metal mixtures on short-term uptake of Cd, Pb, and Zn by rainbow trout (Oncorhynchus mykiss). Aquatic Toxicology **193**:217-227.

Bruslé, J., and G. Anadon. 1996. The structure and function of fish liver. Fish morphology **76**:545-551.

Brzóska, M., and J. Moniuszko-Jakoniuk. 2001. Interactions between cadmium and zinc in the organism. Food Chemical Toxicology **39**:967-980.

Casatta, N., F. Stefani, and L. Viganò. 2017. Hepatic gene expression profiles of a non-model cyprinid (Barbus plebejus) chronically exposed to river sediments. Comparative Biochemistry and Physiology **196**:27-35.

Castaldo, G., M. Pillet, B. Slootmaekers, L. Bervoets, R. Town, R. Blust, and G. De Boeck. 2020. Investigating the effects of a sub-lethal metal mixture of Cu, Zn and Cd on bioaccumulation and ionoregulation in common carp, Cyprinus carpio. Aquatic Toxicology **218**:105363.

Čelechovská, O., Z. Svobodová, V. Žlábek, and B. Macharáčková. 2007. Distribution of metals in tissues of the common carp (Cyprinus carpio L.). Acta Veterinaria Brno **76**:93-100.

Cerqueira, C. C. C., and M. N. Fernandes. 2002. Gill tissue recovery after copper exposure and blood parameter responses in the tropical fish Prochilodus scrofa. Ecotoxicology and Environmental Safety **52**:83-91.

Cho, Y. S., B. N. Choi, K. H. Kim, S. K. Kim, D. S. Kim, I. C. Bang, and Y. K. Nam. 2006. Differential expression of Cu/Zn superoxide dismutase mRNA during exposures to heavy metals in rockbream (Oplegnathus fasciatus). Aquaculture **253**:667-679.

Cinier, C. D., M. Petit-Ramel, R. Faure, and D. Garin. 1997. Cadmium bioaccumulation in carp (Cyprinus carpio) tissues during long-term high exposure: analysis by inductively coupled plasma-mass spectrometry. Ecotoxicology and Environmental Safety **38**:137-143.

Cinier, C. D., M. Petit-Ramel, R. Faure, D. Garin, and Y. Bouvet. 1999. Kinetics of cadmium accumulation and elimination in carp Cyprinus carpio tissues. Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology **122**:345-352.

Cooper, C. A., M. Shayeghi, M. E. Techau, D. M. Capdevila, S. MacKenzie, C. Durrant, and N. R. Bury. 2007. Analysis of the rainbow trout solute carrier 11 family reveals iron import⩽ pH 7.4 and a functional isoform lacking transmembrane domains 11 and 12. FEBS letters **581**:2599-2604.

Couto, N., J. Wood, and J. Barber. 2016. The role of glutathione reductase and related enzymes on cellular redox homoeostasis network. Free radical biology medicine **95**:27-42.

Craig, P. M., C. M. Wood, and G. B. McClelland. 2010. Water chemistry alters gene expression and physiological end points of chronic waterborne copper exposure in zebrafish, Danio rerio. Environmental science & technology **44**:2156-2162.

Custer, T., C. M. Custer, R. Hines, and D. Sparks. 2000. Trace elements, organochlorines, polycyclic aromatic hydrocarbons, dioxins, and furans in lesser scaup wintering on the Indiana Harbor Canal. Environmental Pollution **110**:469-482.

Danabas, D., F. Kutluyer, M. Ural, and M. Kocabaş. 2018. Metal bioaccumulation in selected tissues of barb (Barbus sp.) and common carp (Cyprinus carpio, Linnaeus 1758) from the Keban Dam Lake, Turkey. Toxin Reviews:1-8.

Dautremepuits, C., D. J. Marcogliese, A. D. Gendron, and M. Fournier. 2009. Gill and head kidney antioxidant processes and innate immune system responses of yellow perch (Perca flavescens) exposed to different contaminants in the St. Lawrence River, Canada. Science of The Total Environment **407**:1055-1064.

De Boeck, G., A. Vlaeminck, and R. Blust. 1997. Effects of sublethal copper exposure on copper accumulation, food consumption, growth, energy stores, and nucleic acid content in common carp. Archives of Environmental Contamination and Toxicology **33**:415-422.

De Boeck, G., A. Vlaeminck, P. H. M. Balm, R. A. C. Lock, B. De Wachter, and R. Blust. 2001. Morphological and metabolic changes in common carp, Cyprinus carpio,

during short-term copper exposure: Interactions between Cu2+ and plasma cortisol elevation. Environmental Toxicology and Chemistry **20**:374-381.
De Boeck, G., R. Smolders, and R. Blust. 2010. Copper toxicity in gibel carp Carassius auratus gibelio: importance of sodium and glycogen. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **152**:332-337.
De Boeck, G., T. T. H. Ngo, K. Van Campenhout, and R. Blust. 2003. Differential metallothionein induction patterns in three freshwater fish during sublethal copper exposure. Aquatic Toxicology **65**:413-424.
De Smet, H., B. De Wachter, R. Lobinski, and R. Blust. 2001. Dynamics of (Cd,Zn)-metallothioneins in gills, liver and kidney of common carp Cyprinus carpio during cadmium exposure. Aquatic Toxicology **52**:269-281.
Delahaut, V., B. Rašković, M. S. Salvado, L. Bervoets, R. Blust, and G. De Boeck. 2020. Toxicity and bioaccumulation of Cadmium, Copper and Zinc in a direct comparison at equitoxic concentrations in common carp (Cyprinus carpio) juveniles. bioRxiv:717363.
Díaz-de-Alba, M., A. C. Raya, M. Granado-Castro, M. O. Ramírez, B. El Mai, F. C. García, M. Troyano-Montoro, E. Espada-Bellido, R. T. Santiago, and M. Galindo-Riaño. 2017. Biomarker responses of Cu-induced toxicity in European seabass Dicentrarchus labrax: Assessing oxidative stress and histopathological alterations. Marine pollution bulletin **124**:336-348.
Dickinson, D. A., and H. J. Forman. 2002. Cellular glutathione and thiols metabolism. Biochemical pharmacology **64**:1019-1026.
Eroglu, K., G. Atli, and M. Canli. 2005. Effects of metal (Cd, Cu, Zn) interactions on the profiles of metallothionein-like proteins in the Nile Fish. Bull. Environ. Contam. Toxicol **75**:390-399.
Eyckmans, M., N. Celis, N. Horemans, R. Blust, and G. De Boeck. 2011. Exposure to waterborne copper reveals differences in oxidative stress response in three freshwater fish species. Aquatic Toxicology **103**:112-120.
Ferain, A., C. Bonnineau, I. Neefs, N. De Saeyer, B. Lemaire, V. Cornet, Y. Larondelle, K. A. De Schamphelaere, C. Debier, and J.-F. Rees. 2018. Exploring the interactions between polyunsaturated fatty acids and cadmium in rainbow trout liver cells: a genetic and proteomic study. Aquatic Toxicology **205**:100-113.
Franklin, N. M., J. L. Stauber, R. P. Lim, and P. Petocz. 2002. Toxicity of metal mixtures to a tropical freshwater alga (Chlorella sp.): the effect of interactions between copper, cadmium, and zinc on metal cell binding and uptake. Environmental Toxicology and Chemistry: An International Journal **21**:2412-2422.
Grosell, M. 2011. Copper. Pages 53-133 *in* C. M. Wood, A. P. Farrell, and C. J. Brauner, editors. Fish Physiology. Academic Press.
Grosell, M., and C. M. Wood. 2002. Copper uptake across rainbow trout gills: mechanisms of apical entry. Journal of Experimental Biology **205**:1179-1188.
Grosell, M., C. Nielsen, and A. Bianchini. 2002. Sodium turnover rate determines sensitivity to acute copper and silver exposure in freshwater animals. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **133**:287-303.
Grosell, M., H. J. Hansen, and P. Rosenkilde. 1998. Cu uptake, metabolism and elimination in fed and starved European eels (Anguilla anguilla) during adaptation to water-borne Cu exposure. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology **120**:295-305.
Hamilton, S. J., and P. M. Mehrle. 1986. Metallothionein in Fish - Review of Its Importance in Assessing Stress from Metal Contaminants. Transactions of the American Fisheries Society **115**:596-609.
Handy, R. D. 2003. Chronic effects of copper exposure versus endocrine toxicity: two sides of the same toxicological process? Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology **135**:25-38.

Handy, R., F. Eddy, and H. Baines. 2002. Sodium-dependent copper uptake across epithelia: a review of rationale with experimental evidence from gill and intestine. Biochimica et Biophysica Acta (BBA)-Biomembranes **1566**:104-115.

Hashemi, S., P. S. Kunwar, R. Blust, and G. De Boeck. 2008. Differential metallothionein induction patterns in fed and starved carp (Cyprinus carpio) during waterborne copper exposure. Environmental Toxicology and Chemistry **27**:2154-2158.

Hassan, W., S. Abdullah, M. Afzal, and M. Hussain. 2018. Assessment of acute metals toxicity in Catla catla through hematological and biochemical blood markers. Pakistan Journal of Agricultural Sciences **55**.

Hogstrand, C., and C. Haux. 1990. Metallothionein as an indicator of heavy-metal exposure in two subtropical fish species. Journal of Experimental Marine Biology and Ecology **138**:69-84.

Jakimska, A., P. Konieczka, K. Skóra, and J. Namieśnik. 2011. Bioaccumulation of Metals in Tissues of Marine Animals, Part I: the Role and Impact of Heavy Metals on Organisms. Polish Journal of Environmental Studies **20**.

Jia, X., H. Zhang, and X. Liu. 2011. Low levels of cadmium exposure induce DNA damage and oxidative stress in the liver of Oujiang colored common carp Cyprinus carpio var. color. Fish physiology and biochemistry **37**:97-103.

Kamunde, C., and R. MacPhail. 2011a. Effect of humic acid during concurrent chronic waterborne exposure of rainbow trout (Oncorhynchus mykiss) to copper, cadmium and zinc. Ecotoxicology and Environmental Safety **74**:259-269.

Kamunde, C., and R. MacPhail. 2011b. Metal–metal interactions of dietary cadmium, copper and zinc in rainbow trout, Oncorhynchus mykiss. Ecotoxicology and Environmental Safety **74**:658-667.

Kamunde, C., C. Clayton, and C. M. Wood. 2002. Waterborne vs. dietary copper uptake in rainbow trout and the effects of previous waterborne copper exposure. American Journal of Physiology-Regulatory Integrative and Comparative Physiology **283**:R69-R78.

Kim, J.-H., J.-S. Rhee, J.-S. Lee, H.-U. Dahms, J. Lee, K.-N. Han, and J.-S. Lee. 2010. Effect of cadmium exposure on expression of antioxidant gene transcripts in the river pufferfish, Takifugu obscurus (Tetraodontiformes). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **152**:473-479.

Komjarova, I., and N. Bury. 2014. Evidence of common cadmium and copper uptake routes in zebrafish Danio rerio. Environmental science technology **48**:12946-12951.

Komjarova, I., and R. Blust. 2008. Multi-metal interactions between Cd, Cu, Ni, Pb and Zn in water flea Daphnia magna, a stable isotope experiment. Aquatic Toxicology **90**:138-144.

Komjarova, I., and R. Blust. 2009. Multimetal interactions between Cd, Cu, Ni, Pb, and Zn uptake from water in the zebrafish Danio rerio. Environmental science technology **43**:7225-7229.

Kondera, E., K. Ługowska, and P. Sarnowski. 2014. High affinity of cadmium and copper to head kidney of common carp (Cyprinus carpio L.). Fish physiology and biochemistry **40**:9-22.

Kumai, Y., and S. F. Perry. 2012. Mechanisms and regulation of Na+ uptake by freshwater fish. Respiratory physiology & neurobiology **184**:249-256.

Labbé, S., Z. Zhu, and D. J. Thiele. 1997. Copper-specific transcriptional repression of yeast genes encoding critical components in the copper transport pathway. Journal of Biological Chemistry **272**:15951-15958.

Lange, A., O. Ausseil, and H. Segner. 2002. Alterations of tissue glutathione levels and metallothionein mRNA in rainbow trout during single and combined exposure to cadmium and zinc. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology **131**:231-243.

Laurén, D. J., and D. McDonald. 1987a. Acclimation to copper by rainbow trout, Salmo gairdneri: biochemistry. Canadian Journal of Fisheries and Aquatic Sciences **44**:105-111.

Laurén, D. J., and D. McDonald. 1987b. Acclimation to copper by rainbow trout, Salmo gairdneri: physiology. Canadian Journal of Fisheries and Aquatic Sciences **44**:99-104.

Lee, J., J. R. Prohaska, S. L. Dagenais, T. W. Glover, and D. J. Thiele. 2000. Isolation of a murine copper transporter gene, tissue specific expression and functional complementation of a yeast copper transport mutant. Gene **254**:87-96.

Leung, K. P., D. Chen, and K. M. Chan. 2014. Understanding copper sensitivity in zebrafish (Danio rerio) through the intracellular localization of copper transporters in a hepatocyte cell-line ZFL and the tissue expression profiles of copper transporters. Metallomics **6**:1057-1067.

Lionetto, M., M. Giordano, S. Vilella, and T. Schettino. 2000. Inhibition of eel enzymatic activities by cadmium. Aquatic Toxicology **48**:561-571.

Lodish, H., A. Berk, S. L. Zipursky, P. Matsudaira, D. Baltimore, and J. Darnell. 2000. Osmosis, Water channels, and the regulation of cell volume. Molecular Cell Biology. 4th edition. WH Freeman.

Mackenzie, N. C., M. Brito, A. E. Reyes, and M. L. Allende. 2004. Cloning, expression pattern and essentiality of the high-affinity copper transporter 1 (ctr1) gene in zebrafish. Gene **328**:113-120.

Martinez, C., M. Nagae, C. Zaia, and D. Zaia. 2004. Acute morphological and physiological effects of lead in the neotropical fish Prochilodus lineatus. Brazilian Journal of Biology **64**:797-807.

Matović, V., Z. P. Bulat, D. Djukić-Ćosić, and D. Soldatović. 2010. Antagonism between cadmium and magnesium: a possible role of magnesium in therapy of cadmium intoxication. Magnesium research **23**:19-26.

Matsuo, A. Y., C. M. Wood, and A. L. Val. 2005. Effects of copper and cadmium on ion transport and gill metal binding in the Amazonian teleost tambaqui (Colossoma macropomum) in extremely soft water. Aquatic Toxicology **74**:351-364.

McCormick, S. D. 2001. Endocrine control of osmoregulation in teleost fish. American Zoologist **41**:781-794.

McDonald, D., and C. Wood. 1993. Branchial mechanisms of acclimation to metals in freshwater fish. Pages 297-321 Fish ecophysiology. Springer.

McGeer, J. C., C. Szebedinszky, D. G. McDonald, and C. M. Wood. 2000. Effects of chronic sublethal exposure to waterborne Cu, Cd or Zn in rainbow trout. 1: Iono-regulatory disturbance and metabolic costs. Aquatic Toxicology **50**:231-243.

McGeer, J. C., S. Niyogi, and D. S. Smith. 2011. 3 - Cadmium. Pages 125-184 *in* C.M. Wood, A.P. Farrell, and C. J. Brauner, editors. Homeostasis and Toxicology of Non-Essential Metals, Fish Physiology Part B., Elsevier, New York (2011), pp. 125-184.

Minghetti, M., M. J. Leaver, E. Carpene, and S. G. George. 2008. Copper transporter 1, metallothionein and glutathione reductase genes are differentially expressed in tissues of sea bream (Sparus aurata) after exposure to dietary or waterborne copper. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **147**:450-459.

Niyogi, S., and C. Wood. 2004. Kinetic analyses of waterborne Ca and Cd transport and their interactions in the gills of rainbow trout (Oncorhynchus mykiss) and yellow perch (Perca flavescens), two species differing greatly in acute waterborne Cd sensitivity. Journal of Comparative Physiology B **174**:243-253.

Niyogi, S., S. R. Nadella, and C. M. Wood. 2015. Interactive effects of waterborne metals in binary mixtures on short-term gill–metal binding and ion uptake in rainbow trout (Oncorhynchus mykiss). Aquatic Toxicology **165**:109-119.

Pan, Y.-X., Z. Luo, M.-Q. Zhuo, C.-C. Wei, G.-H. Chen, and Y.-F. Song. 2018. Oxidative stress and mitochondrial dysfunction mediated Cd-induced hepatic lipid accumulation in zebrafish Danio rerio. Aquatic Toxicology **199**:12-20.

Pillet, M., G. Castaldo, S. De Weggheleire, L. Bervoets, R. Blust, and G. De Boeck. 2019. Limited oxidative stress in common carp (Cyprinus carpio, L., 1758) exposed to a

sublethal tertiary (Cu, Cd and Zn) metal mixture. Comparative Biochemistry and Physiology **218**:70-80.

Playle, R. C. 2004. Using multiple metal–gill binding models and the toxic unit concept to help reconcile multiple-metal toxicity results. Aquatic Toxicology **67**:359-370.

Playle, R. C., D. G. Dixon, and K. Burnison. 1993. Copper and cadmium binding to fish gills: estimates of metal–gill stability constants and modelling of metal accumulation. Canadian Journal of Fisheries and Aquatic Sciences **50**:2678-2687.

Pretto, A., V. L. Loro, V. M. Morsch, B. S. Moraes, C. Menezes, B. Clasen, L. Hoehne, and V. Dressler. 2010. Acetylcholinesterase activity, lipid peroxidation, and bioaccumulation in silver catfish (Rhamdia quelen) exposed to cadmium. Archives of Environmental Contamination and Toxicology **58**:1008-1014.

Reynders, H., K. Van Campenhout, L. Bervoets, W. M. De Coen, and R. Blust. 2006a. Dynamics of cadmium accumulation and effects in common carp (Cyprinus carpio) during simultaneous exposure to water and food (Tubifex tubifex). Environmental Toxicology and Chemistry **25**:1558-1567.

Reynders, H., K. van der Ven, L. N. Moens, P. van Remortel, W. M. De Coen, and R. Blust. 2006b. Patterns of gene expression in carp liver after exposure to a mixture of waterborne and dietary cadmium using a custom-made microarray. Aquatic Toxicology **80**:180-193.

Sanchez, W., O. Palluel, L. Meunier, M. Coquery, J.-M. Porcher, and S. Ait-Aissa. 2005. Copper-induced oxidative stress in three-spined stickleback: relationship with hepatic metal levels. Environmental toxicology and pharmacology **19**:177-183.

Sathya, V., M. Ramesh, R. K. Poopal, and B. Dinesh. 2012. Acute and sublethal effects in an Indian major carp Cirrhinus mrigala exposed to silver nitrate: Gill Na+/K+-ATPase, plasma electrolytes and biochemical alterations. Fish & Shellfish Immunology **32**:862-868.

Sevcikova, M., H. Modra, A. Slaninova, and Z. Svobodova. 2011. Metals as a cause of oxidative stress in fish: a review. Vet Med **56**:537-546.

Shaw, B. J., G. Al-Bairuty, and R. D. Handy. 2012. Effects of waterborne copper nanoparticles and copper sulphate on rainbow trout,(Oncorhynchus mykiss): physiology and accumulation. Aquatic Toxicology **116**:90-101.

Sinha, A. K., M. Diricx, L. P. Chan, H. J. Liew, V. Kumar, R. Blust, and G. De Boeck. 2012. Expression pattern of potential biomarker genes related to growth, ion regulation and stress in response to ammonia exposure, food deprivation and exercise in common carp (Cyprinus carpio). Aquatic Toxicology **122**:93-105.

Sinha, A. K., M. Kapotwe, S. B. Dabi, C. D. Montes, J. Shrivastava, R. Blust, and G. De Boeck. 2016. Differential modulation of ammonia excretion, Rhesus glycoproteins and ion-regulation in common carp (Cyprinus carpio) following individual and combined exposure to waterborne copper and ammonia. Aquatic Toxicology 170:129-141.

Skou, J., and M. Esmann. 1992. The Na+, K+-ATPase. Journal of bioenergetics and biomembranes **24**:249-261.

Stewart, A. 1999. Accumulation of Cd by a freshwater mussel (Pyganodon grandis) is reduced in the presence of Cu, Zn, Pb, and Ni. Canadian Journal of Fisheries and Aquatic Sciences **56**:467-478.

Suresh, A., B. Sivaramakrishna, and K. Radhakrishnaiah. 1995. Cadmium induced changes in ion levels and ATPase activities in the muscle of the fry and fingerlings of the freshwater fish, Cyprinus carpio. Chemosphere **30**:367-375.

Taylor, L. N., C. M. Wood, and D. G. McDonald. 2003. An evaluation of sodium loss and gill metal binding properties in rainbow trout and yellow perch to explain species differences in copper tolerance. Environmental Toxicology and Chemistry: An International Journal **22**:2159-2166.

Tellis, M. S., D. Alsop, and C. M. Wood. 2012. Effects of copper on the acute cortisol response and associated physiology in rainbow trout. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **155**:281-289.

Vašák, M. 1991. Metal removal and substitution in vertebrate and invertebrate metallothioneins. Pages 452-458 Methods in enzymology.

Vijayan, M., and T. Moon. 1992. Acute handling stress alters hepatic glycogen metabolism in food-deprived rainbow trout (Oncorhynchus mykiss). Canadian Journal of Fisheries and Aquatic Sciences **49**:2260-2266.

Vinodhini, R., and M. Narayanan. 2008. Bioaccumulation of heavy metals in organs of fresh water fish Cyprinus earpio (Common carp). International Journal of Environmental Science and Technology **5**:179-182.

VMM. 2016. http://geoloket.vmm.be/Geoviews/ (accessed March 2019).

Wang, Q., X. Wang, X. Wang, H. Yang, and B. Liu. 2010. Analysis of metallotionein expression and antioxidant enzyme activities in Meretrix meretrix larvae under sublethal cadmium exposure. Aquatic Toxicology **100**:321-328.

Weber, C. I. 1991. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. 4th ed. EPA/600/4-90/027F. U. S. Environmental Protection Agency, Washington,DC.

Weydert, C. J., and J. J. Cullen. 2010. Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. Nature protocols **5**:51.

Wilson, J. M., P. Laurent, B. L. Tufts, D. J. Benos, M. Donowitz, A. W. Vogl, and D. J. Randall. 2000. NaCl uptake by the branchial epithelium in freshwater teleost fish: an immunological approach to ion-transport protein localization. Journal of Experimental Biology **203**:2279-2296.

Witeska, M., and B. Jezierska. 2003. The effects of environmental factors on metal toxicity to fish (review). Fresenius Environmental Bulletin **12**:824-829.

Wu, P., W.-D. Jiang, Y. Liu, G.-F. Chen, J. Jiang, S.-H. Li, L. Feng, and X.-Q. Zhou. 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 immunology **38**:374-382.

Wu, S., C. Weng, M. Yu, C. Lin, S. Chen, J.-C. Hwang, and P. Hwang. 1999. Cadmium-inducible metallothionein in tilapia (Oreochromis mossambicus). Bulletin of environmental contamination and toxicology **62**:758-768.

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

Zhao, L., Z. Xia, and F. Wang. 2014. Zebrafish in the sea of mineral (iron, zinc, and copper) metabolism. Frontiers in pharmacology **5**:33.

Figure 1

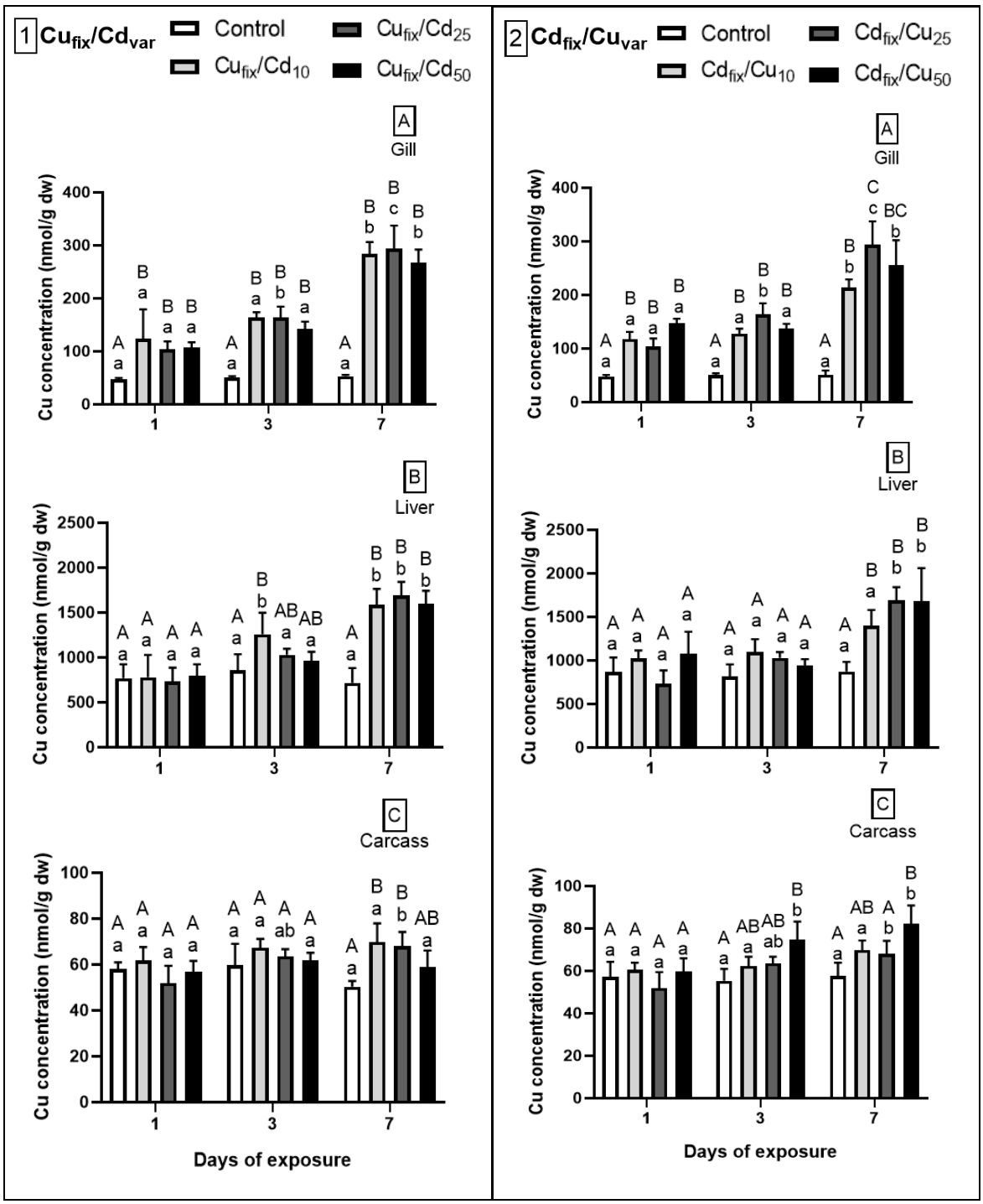

Fig. 1. Copper (Cu) concentration (nmol/g dry weight) in gills (A), liver (B) and carcass (C) of *Cyprinus carpio* exposed to Cu_{fix}/Cd_{var} (1) or Cd_{fix}/Cu_{var} (2) mixtures for 1, 3 and 7 days. Mean ± SD, N=5. Letters were only added when statistical differences occurred. Lower-case letters indicate significant differences ($p < 0.05$) of treatments among sampling days, capital letters indicate significant differences ($p < 0.05$) among treatments within the same sampling day.

Figure 2

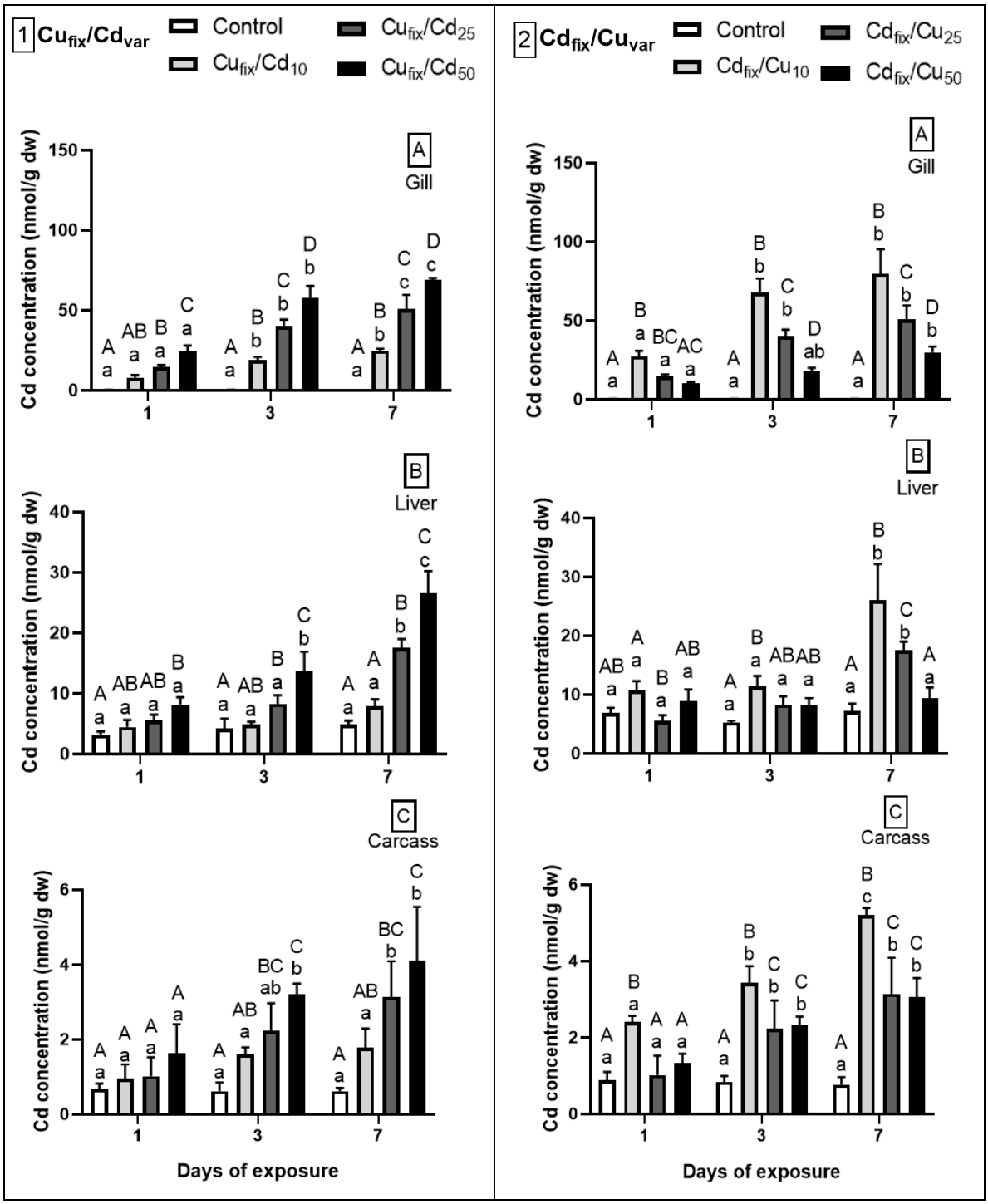

Fig. 2. Cadmium (Cd) concentration (nmol/g dry weight) in gills (A), liver (B) and carcass (C) of *Cyprinus carpio* exposed to Cu_{fix}/Cd_{var} (1) or Cd_{fix}/Cu_{var} (2) mixtures for 1, 3 and 7 days. Mean ± SD, N=5. Letters were only added when statistical differences occurred. Lower-case letters indicate significant differences ($p < 0.05$) of treatments among sampling days, capital letters indicate significant differences ($p < 0.05$) among treatments within the same sampling day.

Figure 3

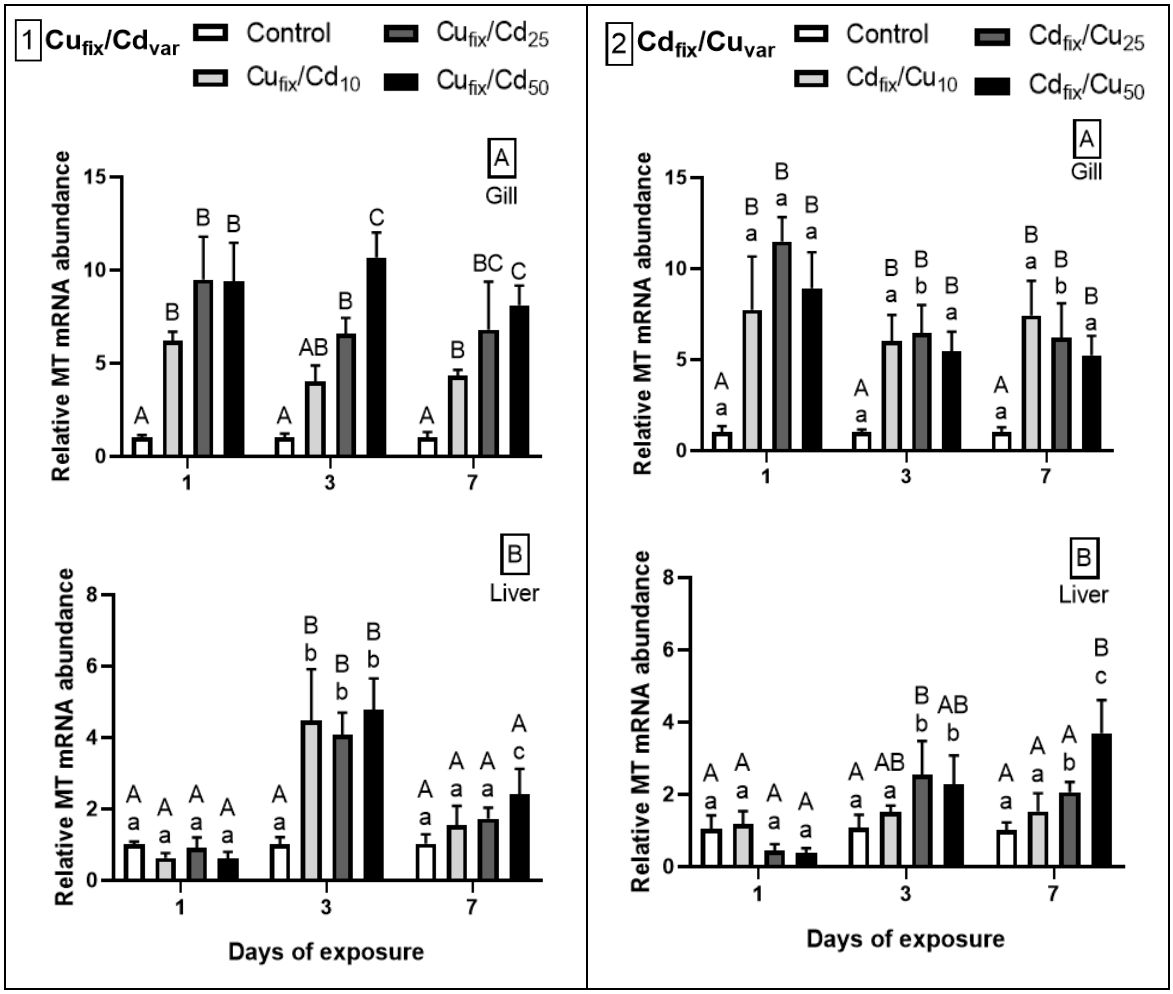

Fig. 3. Relative metallothionein (MT) mRNA abundance in gills (A) and liver (B) of *Cyprinus carpio* exposed to Cu_{fix}/Cd_{var} (1) or Cu_{var}/Cd_{fix} (2) mixtures for 1, 3 and 7 days. Mean ± SD, N=4. Letters were only added when statistical differences occurred. Lower-case letters indicate significant differences ($p < 0.05$) of treatments among sampling days, capital letters indicate significant differences ($p < 0.05$) among treatments within the same sampling day.

Figure 4

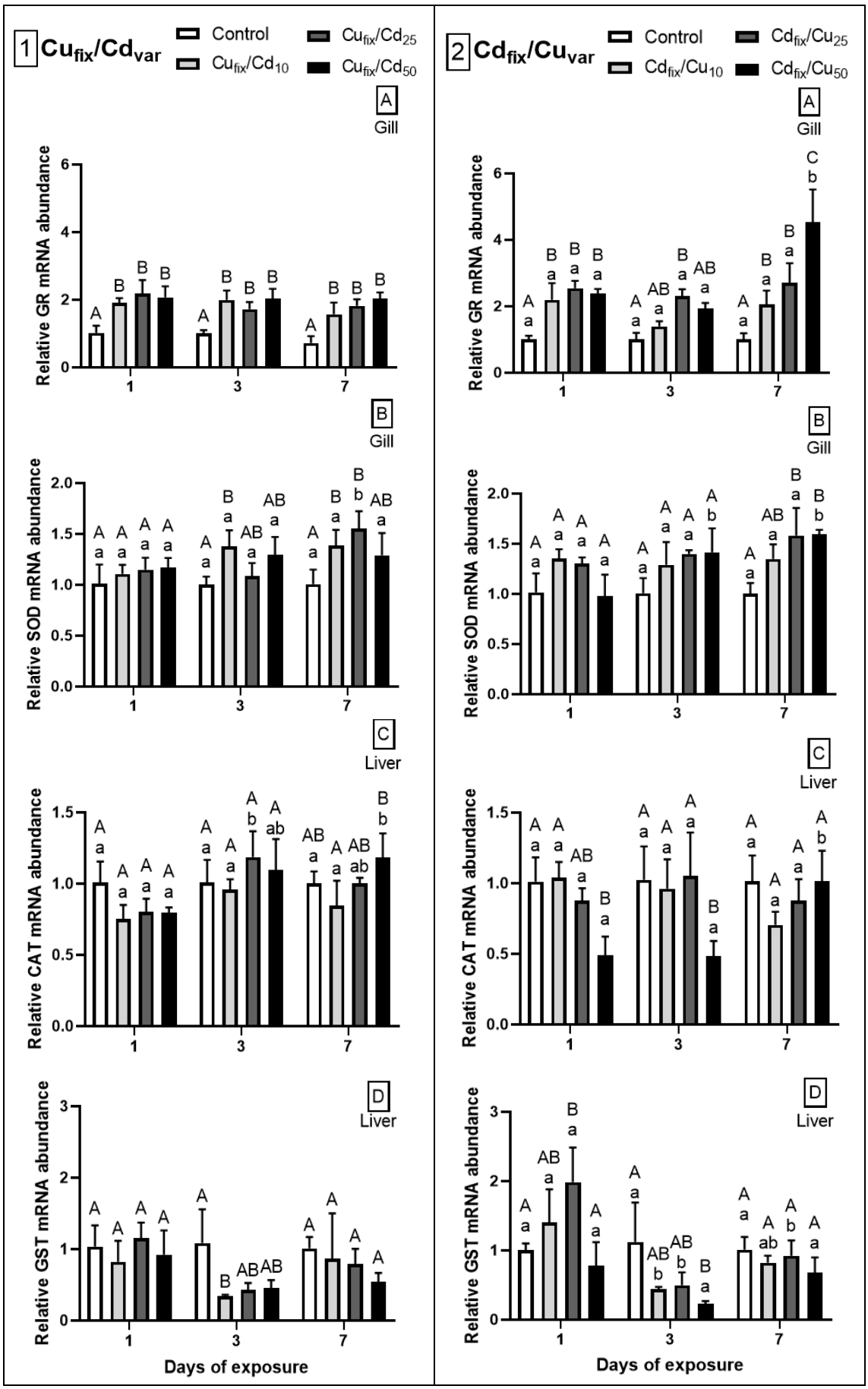
1 Cu$_{fix}$/Cd$_{var}$
Control
Cu$_{fix}$/Cd$_{10}$
Cu$_{fix}$/Cd$_{25}$
Cu$_{fix}$/Cd$_{50}$
2 Cd$_{fix}$/Cu$_{var}$
Control
Cd$_{fix}$/Cu$_{10}$
Cd$_{fix}$/Cu$_{25}$
Cd$_{fix}$/Cu$_{50}$
A Gill
B Gill
C Liver
D Liver
Relative GR mRNA abundance
Relative SOD mRNA abundance
Relative CAT mRNA abundance
Relative GST mRNA abundance
Days of exposure

Fig. 4. Relative glutathione reductase (A), superoxide dismutase Cu-Zn (B), catalase (C) and glutathione S-transferase (D) mRNA abundance in gills and liver of *Cyprinus carpio* exposed to Cu_{fix}/Cd_{var} (1) or Cd_{fix}/Cu_{var} (2) mixtures for 1, 3 and 7 days. Mean ± SD, N=4. Letters were only added when statistical differences occurred. Lower-case letters indicate significant differences ($p < 0.05$) of treatments among sampling days, capital letters indicate significant differences ($p < 0.05$) among treatments within the same sampling day.

Figure 5

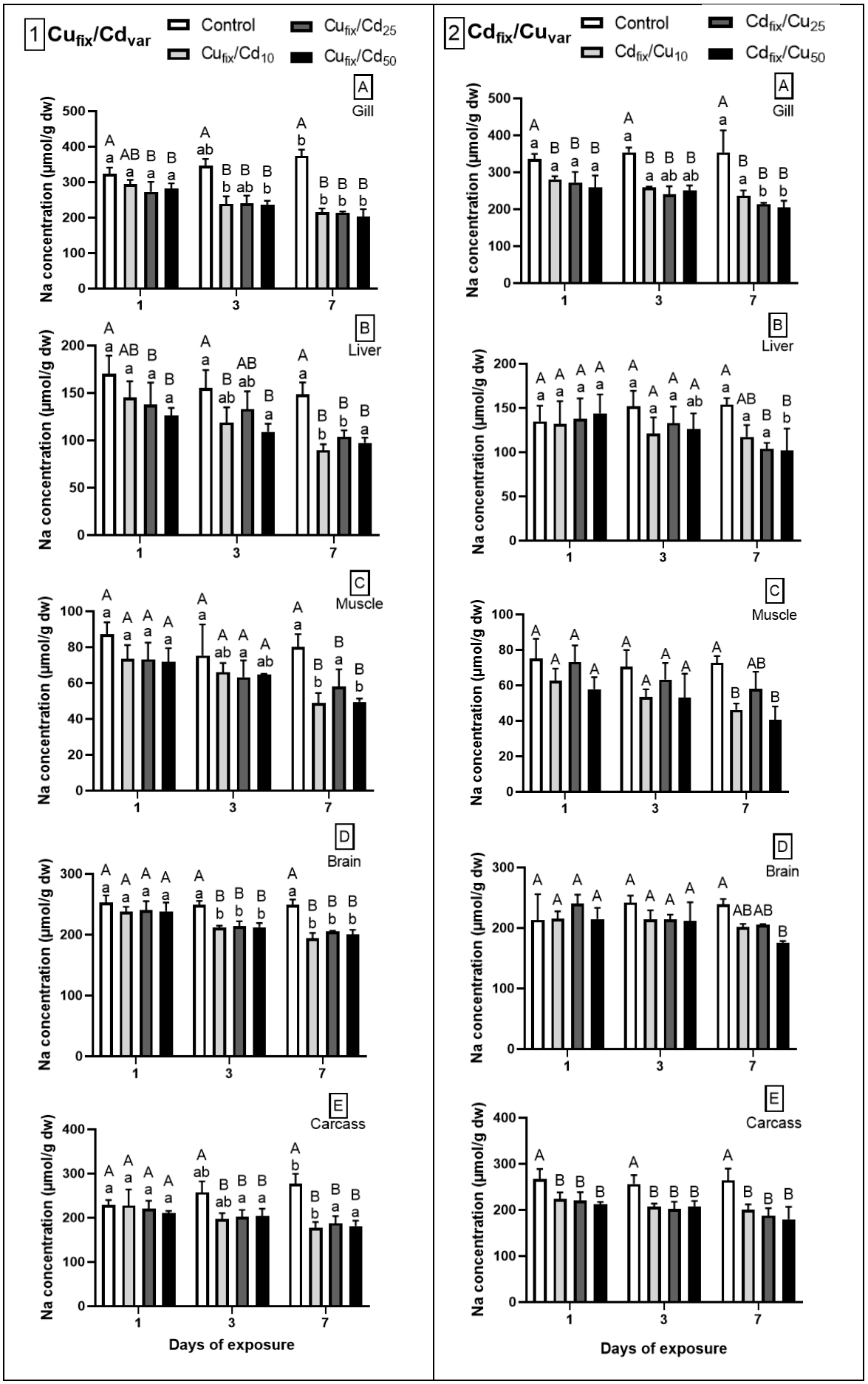
1 Cu$_{fix}$/Cd$_{var}$
Control
Cu$_{fix}$/Cd$_{10}$
Cu$_{fix}$/Cd$_{25}$
Cu$_{fix}$/Cd$_{50}$
2 Cd$_{fix}$/Cu$_{var}$
Control
Cd$_{fix}$/Cu$_{10}$
Cd$_{fix}$/Cu$_{25}$
Cd$_{fix}$/Cu$_{50}$
A Gill
B Liver
C Muscle
D Brain
E Carcass
Na concentration (µmol/g dw)
Days of exposure

Fig. 5. Sodium (Na) concentration (µmol/g dry weight) in gills (A), liver (B), muscle (C), brain (D) and carcass (E) of *Cyprinus carpio* exposed to Cu_{fix}/Cd_{var} (1) or Cd_{fix}/Cu_{var} (2) mixtures for 1, 3 and 7 days. Mean ± SD, N=5. Letters were only added when statistical differences occurred. Lower-case letters indicate significant differences ($p < 0.05$) of treatments among sampling days, capital letters indicate significant differences ($p < 0.05$) among treatments within the same sampling day.

Figure 6

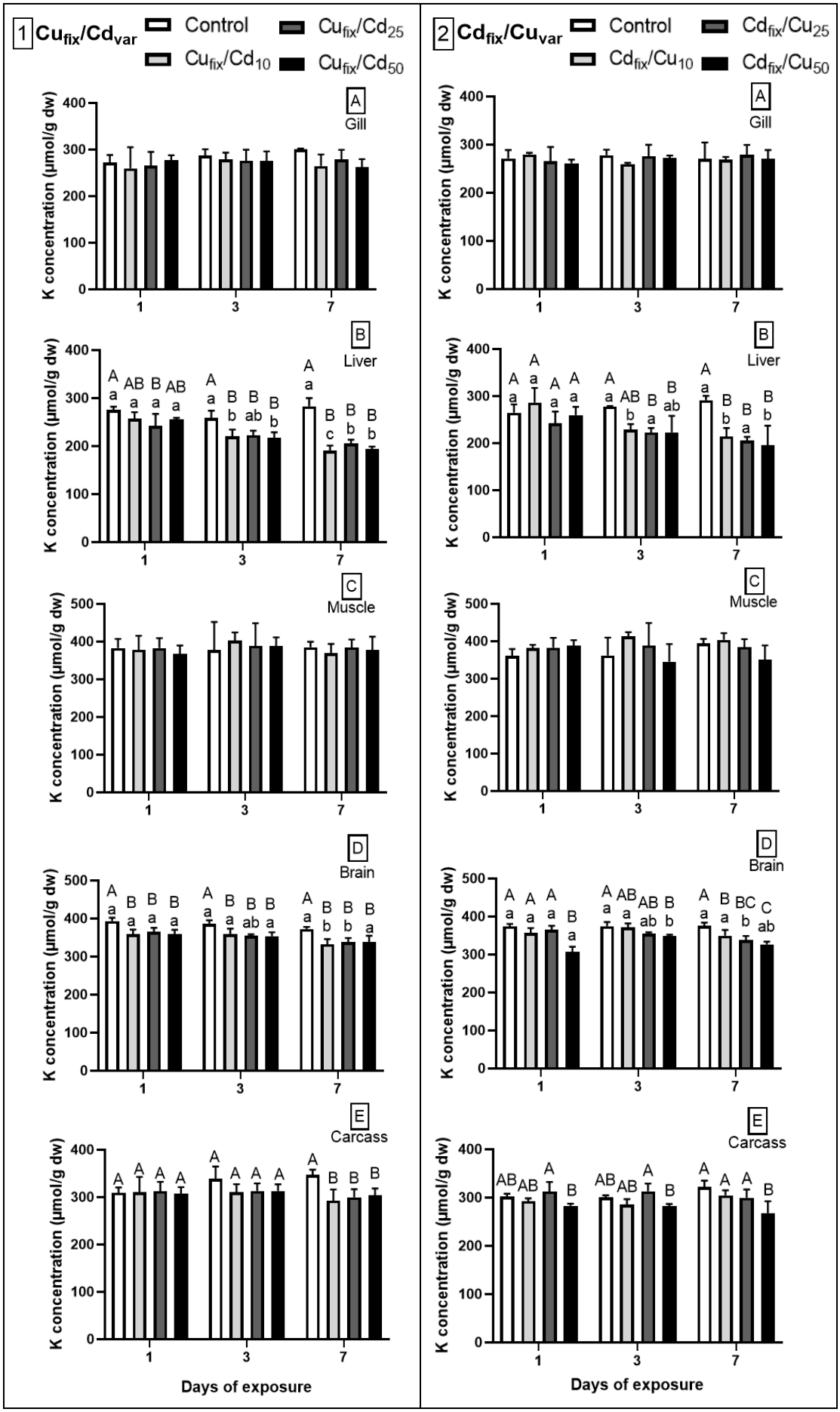
1 Cu$_{fix}$/Cd$_{var}$
Control
Cu$_{fix}$/Cd$_{10}$
Cu$_{fix}$/Cd$_{25}$
Cu$_{fix}$/Cd$_{50}$
2 Cd$_{fix}$/Cu$_{var}$
Control
Cd$_{fix}$/Cu$_{10}$
Cd$_{fix}$/Cu$_{25}$
Cd$_{fix}$/Cu$_{50}$
A Gill
B Liver
C Muscle
D Brain
E Carcass
K concentration (µmol/g dw)
Days of exposure

Fig. 6. Potassium (K) concentration (µmol/g dry weight) in liver (A), brain (B) and carcass (C) of *Cyprinus carpio* exposed to Cu_{fix}/Cd_{var} (1) or Cd_{fix}/Cu_{var} (2) mixtures for 1, 3 and 7 days. Mean ± SD, N=5. Letters were only added when statistical differences occurred. Lower-case letters indicate significant differences ($p < 0.05$) of treatments among sampling days, capital letters indicate significant differences ($p < 0.05$) among treatments within the same sampling day.

Figure 7

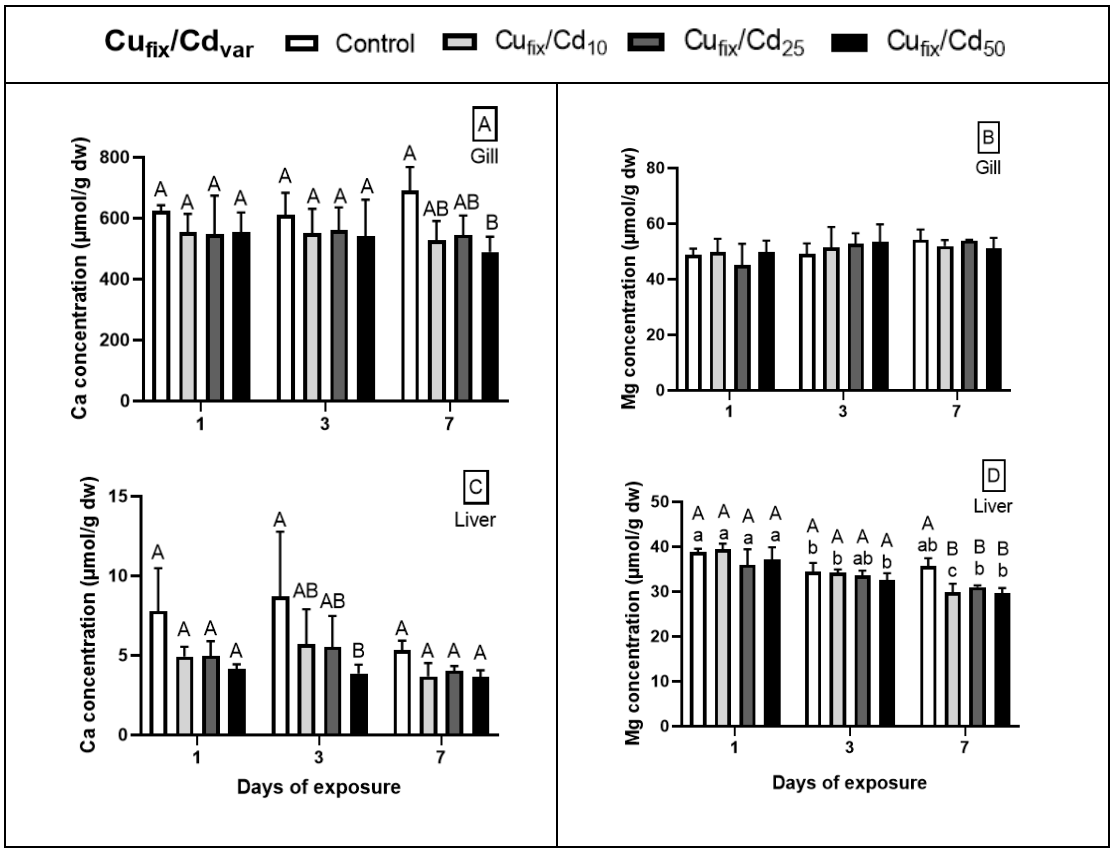

Fig. 7. Calcium and magnesium concentration (µmol/g dry weight) in gills (A,B) and liver (C,D) of *Cyprinus carpio* exposed to Cu_{fix}/Cd_{var} mixtures for 1, 3 and 7 days. Mean ± SD, N=5. Letters were only added when statistical differences occurred. Lower-case letters indicate significant differences ($p < 0.05$) of treatments among sampling days, capital letters indicate significant differences ($p < 0.05$) among treatments within the same sampling day.

Figure 8

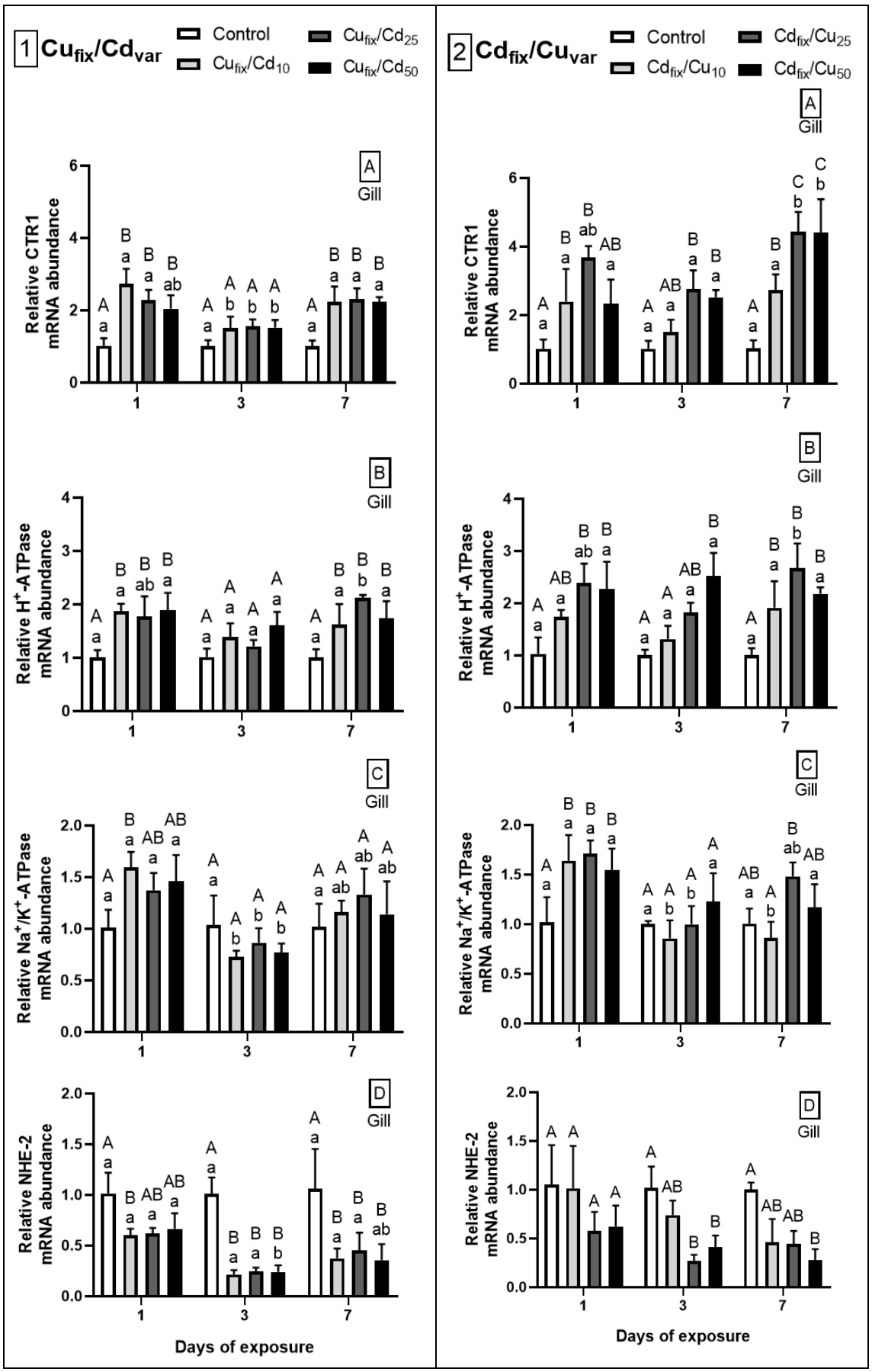

Fig. 8. Relative copper transporter 1 (A), H$^+$-ATPase (B), Na$^+$/K$^+$-ATPase (C) and Na$^+$/H$^+$-exchanger (D) mRNA abundance in gills of *Cyprinus carpio* exposed to to Cu$_{fix}$/Cd$_{var}$ (1) or Cd$_{fix}$/Cu$_{var}$ (2) mixtures for 1, 3 and 7 days.

Mean ± SD, N=4. Letters were only added when statistical differences occurred. Lower-case letters indicate significant differences ($p < 0.05$) of treatments among sampling days, capital letters indicate significant differences ($p < 0.05$) among treatments within the same sampling day.

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Giovanni Castaldo: Conceptualization, Methodology, Investigation, Formal analysis, Writing- Original draft preparation, Writing- Reviewing and Editing**.: Flipkens Gunter**: Conceptualization, Investigation, Formal analysis, Writing - Original draft preparation, Writing- Reviewing and Editing.: **Pillet Marion:** Investigation**,** Methodology**.: Lieven Bervoets**: Supervision, Funding acquisition**.: Raewyn M. Town**: Conceptualization, Validation**.: Ronny Blust:** Funding acquisition, Project administration**.: Gudrun De Boeck:** Conceptualization, Supervision, Funding acquisition, Project administration, Writing- Reviewing and Editing formal analysis.