# Common carp exposed to binary mixtures of Cd(II) and Zn(II) : a study on metal bioaccumulation and ion-homeostasis

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# Aquatic Toxicology


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| **Abstract:** | The aquatic environment receives a wide variety of contaminants that interact with each other, influencing their mutual toxicity. Therefore, studies of mixtures are needed to fully understand their deleterious effects on aquatic organisms. In the present experiment, we aimed to assess the effects of Cd and Zn mixtures in common carp during a one-week exposure. The used nominal waterborne metal levels were 0.02, 0.05 and 0.10 µM for Cd and 3, 7.5 and 15 µM for Zn. Our results showed on the one hand a fast Cd increase and on the other hand a delayed Zn accumulation. In the mixture scenario an inhibition of Cd accumulation due to Zn was marked in the liver but temporary in the gills. For Zn, the delayed accumulation gives an indication of the efficient homeostasis of this essential metal. Between the different mixtures, a stimulation of Zn accumulation by Cd rather than an inhibition was seen in the highest metal mixtures. However, when compared to an earlier single Zn exposure, a reduced Zn accumulation was observed for all mixtures. Metallothionein gene expression was quickly activated in the analysed tissues suggesting that the organism promptly responded to the stressful situation. Finally, the metal mixture did not alter tissue electrolyte levels |
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Highlights

- Common carp were exposed to several binary mixtures of Zn(II) and Cd(II) at fixed and variable concentrations.
- Despite the presence of Zn(II), Cd(II) rapidly accumulated in gills and in liver.
- Zn(II) accumulation was delayed, showing an efficient homeostasis of this metal ion.
- MT gene expression was upregulated to cope with the increased amounts of metals.

# Common carp exposed to binary mixtures of Cd(II) and Zn(II): a study on metal bioaccumulation and ion-homeostasis

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## Abstract

The aquatic environment receives a wide variety of contaminants that interact with each other, influencing their mutual toxicity. Therefore, studies of mixtures are needed to fully understand their deleterious effects on aquatic organisms. In the present experiment, we aimed to assess the effects of Cd and Zn mixtures in common carp during a one-week exposure. The used nominal waterborne metal levels were 0.02, 0.05 and 0.10 µM for Cd and 3, 7.5 and 15 µM for Zn. Our results showed on the one hand a fast Cd increase and on the other hand a delayed Zn accumulation. In the mixture scenario an inhibition of Cd accumulation due to Zn was marked in the liver but temporary in the gills. For Zn, the delayed accumulation gives an indication of the efficient homeostasis of this essential metal. Between the different mixtures, a stimulation of Zn accumulation by Cd rather than an inhibition was seen in the highest metal mixtures. However, when compared to an earlier single Zn exposure, a reduced Zn accumulation was observed for all mixtures. Metallothionein gene expression was quickly activated in the analysed tissues suggesting that the organism promptly responded to the stressful situation. Finally, the metal mixture did not alter tissue electrolyte levels.

Keywords: Mixture stress, Metal pollution, Defence mechanisms, Ion-homeostasis, *Cyprinus carpio*

# 1. Introduction

Trace metals are part of a wide variety of pollutants that have increased in the environment as result of anthropogenic activity (Sevcikova et al. 2011). Moreover, they have a long persistence and can accumulate in the food chain (Eisler 1993, Begum et al. 2005). Even though zinc (Zn) is an essential element, being a key component of structural components and proteins (Watanabe et al. 1997), it can cause problems if present at too high or too low concentrations in the organism. One of the main problems associated with Zn pollution is its ability to lead to disruption of physiological and biochemical mechanisms, one of which is the interference with calcium (Ca) homeostasis (Hogstrand and Wood 1996, Bury et al. 2003, Loro et al. 2014). Cadmium (Cd), in contrast to Zn, is a non-essential metal with no-known biological role (Zheng et al. 2016, Danabas et al. 2018). The toxic effects caused by this metal are related with disruption of ionoregulation (McGeer et al. 2011), oxidative stress and immunosuppression (Zhang et al. 2017).

Both $Cd^{2+}$ and $Zn^{2+}$ ions can compete with each other for their uptake due to their similar chemical characteristics, such as similar size, electron configuration on their outer shell and to their different affinity for the -SH (sulfhydryl) groups, which is greater for $Cd^{2+}$ (Brzóska and Moniuszko-Jakoniuk 2001). It has been shown by Verbost et al. (1988) that $Cd^{2+}$ can interact with the $Ca^{2+}$ transporting ATPase. Similarly, also $Zn^{2+}$ can bind the $Ca^{2+}$ pump, interfering with the transport of this ion (Hogstrand et al. 1996). Moreover, once the metal species are accumulated, they can lead to the production of reactive oxygen species (ROS), causing oxidative stress including lipid peroxidation and osmoregulatory dysfunctions (Livingstone 2001, Zheng et al. 2016). In case of serious oxidative stress, apoptotic events might occur (Pellegrini and Baldari 2009). Apoptosis is induced by intracellular signalling molecules, such as caspase 9 (CASP), which mediates apoptosis through the mitochondrial pathway (Pillet et al. 2019, Wang et al. 2019).

Nonetheless, fish have a suite of defensive mechanisms to cope with increasing ROS and oxidative stress such as the enzyme superoxide dismutase (SOD) which catalyses the conversion of the superoxide radical ($^{\bullet}O_2^-$) into hydrogen peroxide ($H_2O_2$). The $H_2O_2$ is further converted into water ($H_2O$) and oxygen ($O_2$) by catalase (CAT) and glutathione peroxidase (GPx) (Livingstone 2001, Pillet et al. 2019). Furthermore, the presence of peroxiredoxin (Prdx), a family of peroxidases that reduce $H_2O_2$, organic peroxides and peroxynitrite by using cysteine residues helps in protecting the cells and tissues from the effects of oxidant molecules (Tolomeo et al. 2016, Tolomeo et al. 2019).

Moreover, glutathione (GSH) plays a crucial role as a chelating agent for metals (Freedman et al. 1989) and in ROS scavenging (Peña-Llopis et al. 2003). The levels of GSH are ensured by the presence of glutathione reductase (GR), which catalyses the reduction of glutathione disulphide (GSSG) thereby maintaining a constant ratio of GSH/GSSG, and glutathione-S-transferase which metabolizes lipid hydroperoxides (Dautremepuits et al. 2009, Couto et al. 2016). In addition to the antioxidant system, fish utilise metal binding proteins, called metallothioneins (MTs), for protection from metal ion toxicity. The MTs are low molecular weight, cysteine-rich proteins with high affinity for metals (Cretì et al. 2010), which play a key role both in regulation of essential metal ions and sequestration (detoxification) of non-essential metal ions (Amiard et al. 2006). In vitro experiments demonstrated that these proteins exhibit a different binding strength for different metals, following the order $Hg^{2+}$ > $Cu^{+}$ > $Cd^{2+}$ > $Pb^{2+}$ > $Zn^{2+}$ > $Co^{2+}$ (Vašák 1991). Furthermore, MTs can also act as a free radical scavenger (Thornalley and Vašák 1985, Sato and Bremner 1993). This is possible due to the presence of cysteine residues which are oxidized by the scavenging of ROS, such as $H_20_2$ accumulated during oxidative stress (Kumari et al. 1998, Figueira et al. 2012). Furthermore, the induction of MT in aquatic species has been considered as a biomarker for metal pollution (Amiard et al. 2006).

The main aim of this work was to investigate the effects of Cd and Zn mixtures on bioaccumulation, ionoregulation and defensive mechanisms in the common carp, *Cyprinus carpio*, during a short-term exposure (seven days). The nominal concentrations were 0.02, 0.05 and 0.10 µM for Cd and 3, 7.5 and 15 µM for Zn representing respectively 10 %, 25 % and 50 % of the 96h-$LC_{50}$ (the concentration lethal for the 50% of the population) for each metal, as previously determined in our lab (Delahaut et al. 2020). We hypothesized that an antagonistic-like mutual inhibition of Cd and Zn uptake would occur. Furthermore, even though both metals can compete with $Ca^{2+}$ uptake, according to previous results obtained in our lab (Delahaut et al. 2020), we did not expect severe electrolyte loss in tissues. Regarding the defensive mechanisms, we anticipated that metal bioaccumulation would trigger the MT and GR response in order to protect the organism from possible deleterious effects. Lastly, based on the slope of the dose-response curves for each metal (Delahaut et al. 2020), we hypothesized that the metal mixtures would remain sub-lethal.

Finally, in Flanders, the Belgian region where this study was conducted, the water quality guideline for dissolved Cd in rivers and lakes ranges between 0.004 to 0.013 µM (or 0.45 and 1.5 µg/L) according to the water hardness, whereas the value for Zn is set to 0.30 µM (or 20 µg/L) (VLAREM II 2010). Nevertheless, these levels are frequently exceeded. For instance according to a field study done in Flanders over 14 different locations, values for dissolved (filtered through a 0.45 µm membrane) Cd and Zn ranged respectively from 0.001 to 0.20 µM and 1.31 to 33.15 µM (Bervoets and Blust 2003). A more recent publication reported dissolved metal concentrations in two different rivers up to 0.05 µM and 52 µM for Cd and Zn respectively (Michiels et al. 2017), making the metal concentrations range used in this study environmentally relevant.

# 2. Material and methods

## 2.1. Experimental animals

The experimental fish, juvenile common carp (*Cyprinus carpio*), obtained from Wageningen University (Netherlands), were kept for several months at a temperature of 20 ºC with a photoperiod of 12 h light and 12 h dark. Fish were kept in polyethylene (PE) tanks and water quality was ensured by the presence of a biofilter. Three weeks prior to the start of each experiment 250 fish were acclimatized in 200 L of artificial EPA medium-hard water (Weber 1991). The artificial water was prepared by adding salts to deionized water (Aqualab, VWR International, Leuven, Belgium) to generate the following nominal concentrations (VWR Chemicals): $NaHCO_3$ (1.14 mM); $CaSO_4 \cdot 2H_2O$ (0.35 mM); $MgSO_4 \cdot 7H_2O$ (0.5 mM) and KCl (0.05 mM). The calculated water hardness using measured salt concentrations corresponded to 85.6 mg/L $CaCO_3$ (nominal concentration 84.6 mg/L $CaCO_3$). Oxygenation was ensured by the presence of air stones. Experimental methods complied with regulations of Federation of European Laboratory Animal Science Associations (FELASA) were approved by the local ethics committee, University of Antwerp (Permit number: 2015-94, Project 32252).

## 2.2. Experimental set-up

Fish (length = 59.4 ± 4.1 mm; weight = 2.5 ± 0.5 g mean ± standard deviation (SD)) were exposed for one week to two series of waterborne metal mixtures of Cd and Zn ($Cd_{fix}/Zn_{var}$ and $Zn_{fix}/Cd_{var}$). The treatment groups consisted of a fixed concentration of one metal representing 25 % of the 96h-$LC_{50}$ and a variable concentration of the second metal representing 10 %, 25 % and 50 % of the 96h-$LC_{50}$. Five (plus one as backup) double walled polypropylene buckets (PP) for each treatment were used as experimental tanks. Each tank, containing 6 fish, was filled with 9 L of medium-hard water. These experimental tanks were placed in a climate chamber at 20 °C and aerated with an individual air-stone. Water variables were checked daily. The pH and conductivity, measured by the HQ30D Portable Multi-meter (Hach, USA) were respectively 7.93 ± 0.07 and 317 ± 4 µS/cm. In order to avoid build-up of waste products, 8 L (~ 90 %) of water was changed daily and water samples collected to check the stability in metal concentrations. Aerated EPA medium-hard water, used for the water change was prepared 24 h in advance and kept at 20 °C. At day one, three and seven, water samples (N = 108), were collected from the experimental tanks and analysed with the 7700x ICP-MS (Agilent Technologies, Santa Clara, CA, USA) to determine waterborne metal concentrations. The nominal and measured metal concentrations are shown in SI-Table 1. Metal speciation, calculated with the VMinteq software, using measured water parameters is shown in the supplementary information, SI-Tables 2 and 3.

## 2.3. Sampling procedure

Ten fish per treatment (two fish for each tank) were sacrificed at each sampling day (day one, three and seven). Fish were euthanized with an overdose of MS-222 (pH 7.0, ethyl 3-aminobenzoate methane-sulfonic acid, 400 mg/l, Acros Organics, Geel, Belgium). The collected samples were muscle (which represent a large portion of fish biomass), gills (as they are in direct contact with water), liver (important for storage and detoxification), brain (important for neurotoxic and behavioural effects) and the remaining carcasses. The muscle samples were cut near the caudal fin from individual fish. Gill arches and livers were collected from two fish (from the same tank), pooled and divided into aliquots in order to have enough tissue for the analysis. Similar to the gills also the brains and the remaining carcasses were collected and pooled from two fish. All the samples were stored at −80 °C.

## 2.4. Metal bioaccumulation and electrolytes levels

All the samples collected as described above, were stored in pre-weighed Eppendorf bullet tubes, with the exception of the carcasses, which were collected in pre-weighed 50 mL Falcon tubes. Metal and electrolyte content were determined in five samples at each sampling point. In order to check for the accuracy of the procedure six samples of reference material (SRM-2976, mussel tissue, National Institute of Standards and Technology, Gaithersburg, MD, USA) and six blanks were include in the digestion and analysis procedures. Before starting the digestion process, the samples were dried for 48 h, then cooled down in a desiccator for 2 h. After that the dry weight (dw) was recorded with a precision scale (Sartorius SE2, ultra-microbalance). Briefly, the digestion process (Blust et al. 1988, Reynders et al. 2006a) comprised a pre-digestion of 12 h at room temperature with 69 % concentrated $HNO_3$, followed by a microwave digestion. After that, $H_2O_2$ was added to further digest the fat component of the tissue, followed by a final microwave step. Similar to the process, described above, carcasses were also digested using 69 % $HNO_3$ and $H_2O_2$, however the digestion process was carried out using a hot block (Environmental Express, Charleston, SC, USA). At the end of the digestion process, all the samples were diluted to reach a final acid concentration between 1 - 3% with ultrapure Milli-Q (MQ). Metal content and electrolyte levels were determined respectively with 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, ionic charges were only added when relevant for the discussion.

## 2.5. Gene expression

Aliquots of gill and liver samples, collected as described above (~30 - 50 mg), were used for gene expression analysis. Total RNA was extracted according to the manufacturer protocol using Trizol (Invitrogen, Merelbeke, Belgium). In order to determine the quantity and the quality of the RNA, the Nano-Drop spectrophotometry (NanoDrop Technologies, Wilmington, DE) was used. Furthermore RNA integrity was assessed with a 1 % agarose gel with ethidium bromide (500 µg/mL). DNase treatment was performed with the commercial kit DNase I, RNase free kit from Thermo Fisher Scientific (Waltham, MA, USA). The RNA (1 µg) was transcribed to cDNA according to RevertAid H minus First strand cDNA synthesis kit protocol (Thermo fisher, Fermentas, Cambridgeshire). Four samples were selected according to the $OD_{260}/OD_{280}$ and $OD_{260}/OD_{230}$ nm absorption ratios (higher than 1.8 and 2.0 respectively) and used for qPCR. The assay was performed following the Brilliant III Ultra-Fast QPCR Master Mix (Agilent) protocol for Agilent Mx3005P QPCR system in a final reaction volume of 20 µl. The reaction mixture contained 10 µl of Brilliant III Ultra-Fast QPCR Master Mix, 5.7 µl of sterile water, 500 nM of each primer,0.3 µl of reference dye and 5 ng of cDNA. The contamination of reagent was assessed including the "no template" control (e.g. sterile water) in the analysis. The general experimental run protocol as described by Shrivastava et al. (2017), consisted of a denaturation program (3 min at 95ºC), an amplification and quantification program repeated 40 times (15 seconds at 95 ºC, 20 seconds at 60 ºC) followed by a melting curve program (60 ºC– 95 ºC). Oligonucleotides primers were taken from literature: elongation factor 1α (eEF) (Sinha et al. 2012), β-actin (Wu et al. 2014); catalase (CAT) (Wu et al. 2014) , superoxide dismutase Cu-Zn (SOD) (Wu et al. 2014), glutathione reductase (GR) (Wu et al. 2014), metallothionein (MT) (Reynders et al. 2006b), caspase 9 (Casp9) (Pillet et al. 2019). Primers for the glutathione-S-transferase (gst) and glutathione peroxidase (Gpx) were designed using NCBI resources Primer blast and synthesized as highly purified salt-free "OliGold" primers by Eurogentec (Eurogentec, Seraing, Belgium). Quantification cycles (Cq) values were automatically calculated on the log curve for each gene with MxPro qPCR software (Agilent Technologies, Waldbronn, Germany). The stability of the reference genes was tested by two-ways ANOVA both in liver and in the gills. The presence of unique PCR product was assessed by means of the melting curve and the PCR product was verified on agarose gel. The primer efficiency was determined based on the slope of the standard curve. And the relative gene expression determined by means of the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). More information on the primers (e.g. sequence and efficiency) is given in SI-Table 4.

### 2.6. Statistical analysis

All data are presented as mean values ± standard deviation (SD). Before any statistical analysis, all data were checked for normality by the Shapiro-Wilk test. If the data were not normally distributed, they were log transformed. Two-way analyses of variance (ANOVA) were performed on all accumulation and gene expression data, followed by Tukey test. For metal concentration values below the minimum quantification limit (Cd: 0.00089 µM or 0.1 µg/L; Zn: 0.015 µM or 1 µg/L) half of the respective quantification limit values were utilized for the statistical analysis (Custer et al. 2000). In case more than 50% of the observations were BMQL, no statistical tests were conducted. The level of significance for statistical analyses were considered at $p<0.05$. All statistical tests were performed with GraphPad Prism version 8.02 for Windows (GraphPad Software, La Jolla California USA). Data presented in the supplementary information were also analysed using the same software.

## 3. Results

### 3.1. Metal bioaccumulation

#### 3.1.1. Zn bioaccumulation

In fish gills (Fig. 1.1.A and 1.2.A), Zn content showed a similar trend for both the exposure scenarios. However, a significant Zn increase can be observed in the treatment $Cd_{fix}/Zn_{50}$ and $Zn_{fix}/Cd_{50}$ starting from day three. Moreover, also the group $Zn_{fix}/Cd_{10}$ showed a significant increase in Zn compared with the control at day seven (Fig. 1.1.A, 1.2.A). In both the series the metal concentration at day seven was significantly higher compared to day one (Fig. 1.1.A).

As a consequence, the trend in the Zn accumulation rates was similar in both exposure scenarios, i.e. there was almost no Zn accumulation at day one, whereas it starts to increase at day three and seven, especially in fish exposed to the highest Zn concentration (see SI-Tables 5 and 6). Despite the lack of significant differences before day three, Zn accumulation seemed to increase almost linearly in time for both the experimental series (see SI-Fig 1.A and B). In addition, the net accumulated Zn in the series $Cd_{fix}/Zn_{var}$ showed a concentration dependent linear increasing trend for each of the sampling days. In the gills, a limited effect of waterborne Cd on Zn accumulation was noticed by the end of the experiment. In fact, the percentages of Zn increase in the treatment compared to the control were ~ 14, 12 and 30 %, respectively in the $Cd_{fix}/Zn_{10-25-50}$, whereas in the $Zn_{fix}/Cd_{10-25-50}$, the percentage was ~ 21, 12 and 24 %, respectively. Despite the observation in the gills, Zn appears not to accumulate in internal tissues. In fact, in the remaining carcasses (Fig. 1.1.C and 1.2.C), Zn content was almost stable during the whole experiment for both the experimental series, with few differences observed for the treatment $Cd_{fix}/Zn_{50}$ at day seven compared with the same group at day 1 (Fig. 1.1.C) and for the groups $Zn_{fix}/Cd_{25-50}$ respectively at day three and seven, as compared with the same groups at the start of the experiment.

No significant Zn accumulation was observed in the liver (Fig. 1.2.C), or in the remaining tissues (see SI- table 7 and 8).

### 3.1.2. Cd bioaccumulation

Cadmium, in contrast to Zn did accumulate in almost all the analysed tissues. In the gills, Cd showed a fast and continuous increase from day one onwards for both the experimental series (Fig. 2.1.A and 2.2.A). In the series $Cd_{fix}/Zn_{var}$, at day one, fish exposed to the highest concentration of Zn accumulated significantly less Cd compared to the other treatments. However this discrepancy decreased at each sampling day to disappear by the end of the experiment (Fig. 2.1.A). Nonetheless, by the end of the experiment, the percentages of Cd accumulation in the treatment $Cd_{fix}/Zn_{10}$ were, respectively ~ 22 and 15% higher as compared to $Cd_{fix}/Zn_{25-50}$, whereas between $Cd_{fix}/Zn_{25}$ and $Cd_{fix}/Zn_{50}$, there was a difference in Cd accumulation of around the ~ 8%.

In the gills of $Zn_{fix}/Cd_{var}$ exposed fish, a more marked metal accumulation proportional to Cd levels in the water can be observed from the first day of exposure (Fig. 2.2.A). Furthermore, in both the experimental series after one week of exposure, the gill Cd content in all treatments significantly increased as compared to the previous sampling day (Fig. 2.1.A and 2.2.A). Cadmium gill accumulation in the $Cd_{fix}/Zn_{var}$ series showed an almost linear increase over time (see SI-Fig 2.A). Looking at the accumulation rates, an inhibition of accumulated Cd by Zn levels can be observed only at day one and three, in fish exposed to the highest waterborne Zn level (See SI-Table 5). In the $Zn_{fix}/Cd_{var}$ scenario, Cd accumulation increased both through time and among the different exposure levels without reaching steady-state (see SI-Fig 2.B and C), showing an accumulation-rates pattern corresponding to waterborne Cd exposure levels (See SI-Table 6).

In the liver, Cd concentrations increased from day one onwards in nearly all the treatments for both experimental series, (Fig. 2.1.B and 2.2.B). Moreover, significant differences between treatment groups were also observed after one day of exposure and became more evident by the end of the experiment. In the series $Cd_{fix}/Zn_{var}$, carp exposed to the highest waterborne Zn concentration accumulated significantly less Cd in their liver (Figure 2.1.B), whereas in the $Zn_{fix}/Cd_{var}$ series, Cd accumulation increased with increasing waterborne Cd levels (Fig.

2.2.B). By the end of the experiment, the metal content in all the treatment groups, for both the experimental series, was higher as compared with the previous days (Fig. 2.1.B and 2.2.B).

In the remaining carcasses, a significant increase in Cd content compared to the control groups, for both the experimental series, can be observed in all the treatments from day one onwards (Fig. 2.1.C and 2.2.C). The treatment $Cd_{fix}/Zn_{50}$, accumulated less Cd compared to the treatment $Cd_{fix}/Zn_{10}$ at day one and three (Fig. 2.1.C). In the series $Zn_{fix}/Cd_{var}$, an increasing accumulation trend reflecting waterborne Cd concentrations can be observed from day one onwards (Fig. 2.2.C). For both the experimental series, almost all the treatments accumulated more Cd, compared with the same groups at the previous sampling day (Fig. 2.1.C and 2.2.C).

In both the exposure series, metal concentrations in the muscle stayed below the minimum quantification limit during the whole experiment, whereas in the brain Cd was detected only in few samples and mostly by the end of the experiment (see SI- table 7 and 8).

## 3.2. Gene expression

### 3.2.1. Metallothionein

An increased expression compared to the control of the gene coding for MT can be observed in nearly all the treatments from the first day onwards, in both the exposure series (Fig. 3.1.A and 3.2.A). In addition, this increase lasted until the end of the experiment. The expression of the MT gene in the in treatment $Cd_{fix}/Zn_{50}$ significantly increased at day three compared to the previous day, however after one week no further differences were noticed compared to the previous sampling days (Fig. 3.1.A). In the liver, a significant induction of MT mRNA compared to the control was observed in the treatments $Cd_{fix}/Zn_{25-50}$ at day three. However, the treatment $Cd_{fix}/Zn_{25}$ returned to the control levels at day 7, whereas the gene expression of the treatment $Cd_{fix}/Zn_{50}$ showed a further increase as compared with the previous day (Fig. 3.1.B). In the exposure series $Zn_{fix}/Cd_{var}$, a significant induction of the MT mRNA was observed only in treatment $Zn_{fix}/Cd_{50}$ from day three onwards (Fig. 3.2.B).

### 3.2.2. Antioxidant enzymes

No significant differences were observed regarding the GR gene expression between control and treatment groups in the gills of the exposure series $Cd_{fix}/Zn_{var}$ (Fig. 4.1.A). In the second exposure scenario an induction of the gene coding for the GR occurred at day seven for the group $Zn_{fix}/Cd_{50}$ as compared to the control (Fig. 4.2.A). The hepatic expression of the GR in both the exposure scenarios showed similar levels between control and treatment groups (Fig. 4.1.A and 4.2.A). Regarding the GST mRNA abundance, even though an increasing trend can be observed in the liver of fish exposed to variable concentrations of Cd at day three, no differences were observed between control and treatment groups during the whole experiment in both the analysed tissue (Fig. 4.1.B and 4.2.B). No statistically significant changes between controls and treatments, in both the exposure scenarios were observed for the remaining genes (see SI- table 9 and 10)

### 3.2.3. Indicator of apoptosis

Regarding caspase 9 gene expression, no differences were observed in the gills between control and treatments for both the experimental series (Fig. 5.1.A and 5.2.A). In the liver, the CASP gene in the treatment $Cd_{fix}/Zn_{25-50}$ was significantly induced compared to the control after one week of exposure (Fig. 5.1.B). In the exposure series $Zn_{fix}/Cd_{var}$, increased gene expression can be observed in the treatments $Zn_{fix}/Cd_{10-50}$ at day seven compared to the control (Fig. 5.2.B).

## 3.3. Tissue electrolyte levels

Calcium concentrations in the gills and in the carcasses are shown in Fig. 6. In both exposure series, Ca levels in the gills did not show differences between control and treatment groups (Fig. 6.1.A and 6.2.A). In the remaining carcasses in the series $Cd_{fix}/Zn_{var}$, no differences were observed between control and treatment (Fig. 6.1.B), whereas, in the exposure scenario $Zn_{fix}/Cd_{var}$, the Ca concentrations in the treatment $Zn_{fix}/Cd_{25}$, showed lower Ca levels compared to the control at day seven (Fig. 6.1.B). Calcium levels in the muscle of fish exposed to $Cd_{fix}/Zn_{var}$ showed some differences in the treatment (e.g. $Cd_{fix}/Zn_{25}$ at day three and $Cd_{fix}/Zn_{50}$ at day seven) as compared to the control, although this seems to be due to an internal variation such as increased Ca levels in the control at day seven as compared to day one (≃ 42%) (see SI-table 7 and 8).

Regarding Mg, lower electrolyte values were reported at day seven in the treatments $Cd_{fix}/Zn_{25}$ and $Zn_{fix}/Cd_{25\text{-}50}$ compared to the control group in the gill tissue (see SI-table 7 and 8).

No differences were observed between control and treatment groups for Na or K (see SI-table 7 and 8).

# 4. Discussion

We hypothesized that metal bioaccumulation would take place in fish exposed to waterborne Cd-Zn mixtures and, as a consequence, that an induction of defensive mechanisms would occur. Our results showed on the one hand a delayed Zn accumulation and on the other hand a sharp Cd increase. Nonetheless common carp were able to cope with the level of stress caused by metal ions by minimizing adverse effects; in fact, no mortality was reported during the whole experiment.

## 4.1. Metal bioaccumulation

### 4.1.1. Zinc and cadmium bioaccumulation in the gills

Zn accumulation occurred only in the gills and, as expected from previous studies (Castaldo et al. 2020, Delahaut et al. 2020), showed a delayed accumulation. In contrast, Cd accumulated quickly and in several internal tissues. This difference between Zn and Cd bioaccumulation is no surprise considering that fish can adjust a number of transporters and regulate uptake/excretion mechanism in order to control the metal accumulation (Hogstrand et al. 1995, Hogstrand et al. 1996).

Considering the net branchial accumulated values in the binary mixture, it seems that the predicted inhibitory effect of Cd on Zn accumulation was not clear, which is perhaps no surprise as Cd levels were at least 57 times lower (from 0.026 to 0.126 µM Cd). On the contrary, Zn accumulation seemed to be slightly stimulated at the highest Cd concentration. In Nile tilapia (*Oreochromis niloticus*) exposed to 1 ppm of Zn plus 0.1 ppm of Cd, accumulated gill Zn levels were similar to values observed when exposed to Zn alone, whereas when exposed to 10 ppm Zn plus 1 ppm Cd, a stimulation of Zn accumulation occurred (Kargın and Çoğun 1999). In the mussel *Mytilus edulis planulatus* exposed to several metal mixtures of Cu ( 10 - 20 µg/L or 0.15 – 0.30 µM), Cd (10 – 20 µg/L or 0.088 or 0.17 µM) and Zn (100 – 200 µg/L or 1.5 – 3 µM), an increased Zn accumulation was observed in the presence of either Cu or Cd, although in the latter case the increased Zn accumulation happened only if Cd or Zn were at the highest concentrations (Elliott et al. 1986). Cadmium in contrast to Zn, showed an accumulation trend that followed the waterborne Cd levels in the exposure scenario $Zn_{fix}/Cd_{var}$. However, in the series $Cd_{fix}/Zn_{var}$, Cd accumulation in the gills appeared to be slightly reduced by the presence of waterborne Zn, at least for the first three days of the experiment. It is known that Cd can enter the gills via Ca channels (Verbost et al. 1989) and that fish have the ability to reduce the affinity for $Ca^{2+}$ transporters (Hogstrand et al. 1995). Therefore, one might link this trend to the ability of fish to reduce the affinity for transporters in order to reduce metal uptake. Thus Cd might have entered via other channels, such as the divalent metal transporter (DMT1) (Komjarova and Bury 2014).

When comparing the net accumulated metal values in the mixtures with the ones obtained in the single exposure scenarios (Castaldo et al. 2020, Delahaut et al. 2020), some antagonistic-like effects on the uptake of the two metals can be noticed. In fact, the net-accumulated metal concentrations in common carp exposed to 10, 25 and 50% of the 96h-$LC_{50}$ in a single exposure scenario after seven days were, respectively ≃ 4.61, 5.36 and 8.80 µmol/g dw for Zn and 90, 137 and 267 nmol/g dw for Cd. Therefore, in the mixture, the presence of a fixed concentration of Cd led to a Zn accumulation reduction ranging from ≃ 55 % to 70 %. However, it should be mentioned that in the single exposure scenario, the control group at day seven had less Zn compared to same group at day one, explaining partially the high net accumulation. If we correct for this variation and calculate the net values at day seven using the control values obtained at day one, the net Zn accumulation for the 25 and 50% of the 96h-$LC_{50}$ were ≃ 2.26 and 5.7 µmol/g dw respectively. Even then, an antagonistic-like effect of Cd on Zn bioaccumulation was still present. Even though by the end of the experiment the presence of Zn appeared not to inhibit the branchial Cd uptake, during the first days of exposure the accumulated metal levels were lower in the mixture. Moreover, Cd levels decreased as Zn in the water increased. Using data from Van Ginneken et al. (1999) to estimate Cd uptake in a competitive interaction scenario with Zn under our waterborne metal concentrations, the inhibitory effect of Zn on Cd uptake probably already started after 3 hours of exposure.

Inhibitory effects between the two metals on the their respective uptake were pointed out by several authors. For example, Fırat et al. (2009) found lower branchial and hepatic Zn levels in Nile tilapia exposed to a mixture of Zn and Cd as compared with fish exposed to individual metals. Similarly, Saibu et al. (2018) found that Zn accumulation in the gills was highly reduced in presence of Cd, suggesting a competitive interaction between these two metals at the uptake site. Moreover, in zebrafish (*Danio rerio*) Komjarova and Blust (2009) showed that the uptake of Cd was reduced by the presence of Zn.

Therefore, comparing both the results obtained in the single exposure scenario and in the binary mixture, one can assume that in common carp, the metals do play an inhibitory role on the accumulation of the other metal, albeit a relatively small one. Furthermore, the results obtained at the end of the experiment, at higher waterborne Cd levels, might possibly indicate gill damages at these Cd levels. Nevertheless, more detailed studies using isotopic forms of the metals to differentiate newly accumulated from background metals, are needed to validate these thoughts.

Finally, the difference observed in the mixtures between Zn and Cd accumulation can be linked with the higher affinity that Cd has for gill binding sites as compared to Zn (Playle et al. 1993, Playle 2004). Specifically, Cd binds to the gills approximately 1000 times stronger than Zn under equal exposure conditions (Playle, 2004). Moreover considering that both Zn and Cd have high affinity for cysteine protein in the order Cd > Zn (Saibu et al. 2018), is reasonable to assume that Cd displaced the Zn bound to these protein, which was subsequently flushed away.

### 4.1.2. Metals bioaccumulation in the remaining tissues

It is known that the metal concentration changes are a result of uptake and excretion processes, thus the Zn observations, not only in the gills but also remaining tissues and carcasses, can be related to its homeostasis. In fact Zn homeostasis is strictly controlled at both organismal and cellular level (Bury et al. 2003). For example, rainbow trout exposed to 2.3 µM of Zn can reduce, after seven days of exposure, the affinity of $Ca^{2+}$ carriers (increasing the Km) in order to decrease the branchial $Zn^{2+}$ influx (Hogstrand et al. 1995). Nevertheless, fish were able to restore the $Ca^{2+}$ transporting capacity (*Jmax*) in order to maintain Ca homeostasis in the plasma even with a decreased affinity for the transporting sites (Hogstrand and Wood 1995). Moreover, in zebrafish, Zn supplementation resulted in an increased expression of the Zn exporter ZnT1 and in a decreased expression of the ZIP importer ZIP10 (Hogstrand et al. 2008). The ZIP proteins are a family of proteins involved in the uptake and transport of Zn into the cytosol (Hogstrand 2011). Furthermore, the transcript abundance of some ZIP proteins, such as the ZIP8 can also be affected by different metal mixtures. For example, Komjarova and Bury (2014) found that in zebrafish, a Cd, Cu mixture (0.025 µM Cd plus 0.5 µM Cu) significantly reduced the ZIP8 transcript, compared to Cd and Cu exposure alone. In the case that Cd uptake occurs via this transporter, like in mice (Dalton et al. 2005), the authors suggested that this decrease may partially explain the reduction in Cd transport.

In the liver Cd accumulated quite rapidly in both exposure scenarios. In the series $Zn_{fix}/Cd_{var}$, the Cd bio-accumulation reflected waterborne Cd concentrations and the accumulation pattern observed in the gills. Similar to our findings, a Cd accumulation inhibition due to Zn was also reported in the liver of Nile tilapia exposed to Cd (1 mg/L or 8.16 µM) plus Zn (5 mg/L or 76 µM) and fathead minnow (*Pimephales promelas*) exposed to Cd, Zn mixture (0.05 µM of Cd plus 3 µM of Zn) (Fırat et al. 2009, Driessnack et al. 2017). In the $Cd_{fix}/Zn_{var}$ series, the inhibitory effects of Zn on Cd accumulation were more marked compared to those observed in the gills. This reflects that the gills, being in direct contact with the external media are the primary uptake site for metal ions (Heath 1995) and after the Cd has been taken up by the organism, it is transported to the liver and kidneys (Olsson et al. 1998) where excretion processes take place. However on a mass balance basis, Cd excretion via both the kidney and the bile is low in relation to Cd uptake and accumulation (McGeer et al. 2011). Furthermore as shown by (Handy 1996), a small portion of Cd can be excreted by the gills.

Looking at the results, both for Zn and Cd in either the exposure scenarios, one can assume that common carp is able to regulate Zn uptake and excretion processes well. This was at least the case in fish exposed to the lowest Zn concentration for seven days and to a lesser extent in fish exposed to 25 and 50 % of the $LC_{50}$, where eventually some accumulation occurred. For Cd, the metal increase in the remaining carcasses, which occurred concomitantly to the one in the liver might suggest that excretory mechanisms were struggling to compensate for metal uptake. Nonetheless it is worth to mention that Cd levels in the muscle remained below the detection limit, thus the above mentioned increase for the carcass might be linked with metal absorbed by the skin and in the remaining organs (e.g. eyes and kidney). Finally, the fact that Cd levels in the brain were detected only by the end of the experiment in a limited number of samples seems reasonable considering that it is protected by the blood brain barrier, which can prevent the accumulation of toxic substances such as Cd (Szebedinszky et al. 2001).

## 4.2. Defensive mechanisms and indicators of apoptosis

Metallothioneins are cysteine rich proteins which play a crucial role in essential metal homeostasis and in the detoxification of non-essential metals (Hamilton and Mehrle 1986, De Boeck et al. 2003). In our experiment a fast and long lasting MT gene induction occurred in the gills during both the experimental series, whereas in the liver the induction of the gene was delayed to day three. In our experiment Zn levels remained almost stable during the first days of exposure, whereas Cd levels increased. Probably Cd displaced at least some of the Zn from the cysteine binding sites, allowing Zn to be flushed away, although one has to keep in mind that Cd levels are lower compared to Zn levels. Waalkes et al. (1984), showed with an in vitro experiment that the displacement of Zn by Cd occurs with an $EC_{50}$ (effect concentration that displace the 50% of bound Zn) of around 1.33 µM. However, Cd seemed to produce less of an increase in hepatic MT at equitoxic concentrations compared to Zn (Waalkes et al. 1984).

The differences in MT induction observed between the two tissues, might be linked with the MT background levels, which are higher in the liver compared to the gills (Hashemi et al. 2008), and thus might be sufficient to cope with the accumulated metals. Nonetheless, as hypothesized by several authors, the involvement of cytoplasmatic foci, such as the stress granules (Ferro et al. 2015, Chatzidimitriou et al. 2020, Ferro et al. 2020), in which the mRNA is stored for future translation (Lavut and Raveh 2012) can not be excluded. Furthermore as observed in juvenile rainbow trout, even though Cd accumulation on molar basis was not tracked quantitatively by the MT induction, the levels of this protein present in the liver and in the kidney were adequate to complex all the accumulated Cd, whereas this was not the case in the gills (Hollis et al. 2001). This increase in MT gene expression increase can be thought of as a “state of readiness”, considering that metals are transferred to storage and excretion organs such as liver (Cinier et al. 1999, Arini et al. 2015). In fact as demonstrated by Arini et al. (2015) MTs synthesized in response to a metal exposure can be maintained for several weeks at cellular level. This would clearly represent an advantage in case of persistent metal contamination and higher levels of accumulated metals. This thought appears to be in line with the presence of stress granules, which by stabilizing the mRNA contained in them will allow a faster response to stress. Some recent studies on fish focused on the gene expression of stress granule nucleation proteins seems to confirm this hypothesis (Nicorelli et al. 2018).

As already mentioned, besides MT, fish have various antioxidant enzymes to cope with ROS (Wang et al. 2010). However, in the current study, despite the accumulated metal levels, the only variation was observed for GR by the end of the experiment in the treatment $Zn_{fix}/Cd_{50}$. Knowing that common carp rely on GSH which can bind metal ions as first line of defence, this GR increase can be interpreted as an attempt of the fish to reduce the oxidized glutathione and increase the free radical scavenging ability of the cells (Eyckmans et al. 2011). Considering the late response in GR and the lack of changes for the other analysed genes, one might assume that the metal levels stayed below the threshold to significantly induce ROS production. However it is worth to mention that some signs of apoptosis signalling were observed in the liver of common carp by the end of the experiment. Apoptosis is a regulatory process involved in the destruction of damaged cells (Gao et al. 2013), and as suggested by Pillet et al. (2019), an increase in caspase could be an attempt to destroy damaged cells in order to avoid more deleterious effects. Thus the failure to increase the gene expression of antioxidant enzymes might be linked, as mentioned above, with the role of stress granules. Furthermore it is worth to mention the ROS scavenging role played by the MTs due to their cysteine-thiol groups (Thornalley and Vašák 1985, Sato and Bremner 1993). In fact previous studies in organisms exposed to metals, reported increased levels of oxidized MTs (Santovito et al. 2008).

Overall, the obtained results suggest that common carp were able, at least for one week, to cope with adverse effects caused by these metal ions. Moreover, the analysis of caspase 9 gene expression in metal mixtures can be considered as an interesting approach to further investigate apoptotic processes, although these pathways are complex and cannot be explained by changes in caspase gene expression alone.

## 4.3 Electrolyte levels

Electrolytes (e.g. $Na^+$, $K^+$, $Ca^{2+}$) are important for physiological and metabolic processes (Sathya et al. 2012). In the present study, no substantial differences were observed in electrolyte content. For instance, Na and K levels in the present study were not impacted by the metal mixture. Similarly, even though both Cd and Zn are known to be $Ca^{2+}$ antagonists, competing with it at the uptake site and inhibiting the $Ca^{2+}$-ATPase (Hogstrand 2011, McGeer et al. 2011), no gill Ca loss occurred. Several studies reported a Ca loss in freshwater fish exposed to Cd and Zn, such as rainbow trout and killifish (*Fundulus heteroclitus*) (McGeer et al. 2000, Loro et al. 2014). Nonetheless, in the carcasses and the muscle some Ca loss was observed. However, this apparent loss appears to be more related with biological variation rather than with the metal exposure. Similarly, also the few differences observed for Mg (e.g. gills and carcasses) seems more due to internal variation rather than the metal exposure. The lack of effects of the metals on Mg content, seems to be in line with Reynders et al. (2006a), who found no changes in plasma Mg content in common carp simultaneously exposed to Cd via water (≃ 0.08, 0.93 and 4 µM) and via food (≃ 0.08, 1.08 and 1.26 µM).

Generally, metal toxicity decrease with the increasing of water hardness, due to competition between metal ions and $Ca^{2+}$ and $Mg^{2+}$ ions (Kim et al. 2001, Pyle et al. 2002, Ebrahimpour et al. 2010), thus the lack of effects on electrolyte levels could be attributed to the protective role that ambient Ca play towards metal toxicity (Hollis et al. 2000), to the relatively low waterborne metal concentrations and to the short exposure period.

## 5. Conclusions

The main goal of the present study was to assess the effects of binary waterborne metal mixtures on bioaccumulation, defensive mechanisms, ion-homeostasis and survival rate in common carp. Our main hypothesis was that metal accumulation would occur to a different extent for Zn and Cd. In addition, an antagonistic-like effect on the accumulation between the two metals was expected. As predicted, Zn accumulated quite slowly in the gills, whereas Cd accumulation was fast and occurred since day one for all the treatments. Looking at Zn accumulation in the binary mixture the predicted antagonistic-like effect is not clear, but it becomes more evident when comparing with previous exposure studies. In contrast with our hypothesis, no accumulation of Zn occurred in the remaining tissues. Regarding Cd accumulation, as predicted, a fast and sharp accumulation occurred in the gills, liver and carcasses. In the gills, the anticipated inhibition of Zn on Cd accumulation rate was evident during the first days of exposure, but disappeared thereafter. In the liver the antagonistic-like effects between the two metals became more evident as time passed. Metallothionein gene expression was continuously upregulated in the gills in order to mitigate possible deleterious effects. As expected no significant changes due to the metal exposure occurred in electrolyte levels. As previously mentioned, it is likely that toxic effects of metals were counteracted by water hardness and $Ca^{2+}$ levels in the exposure media. Our final hypothesis, confirmed by the lack of mortality, was that the metal mixture remained sub-lethal. In conclusion, we can affirm that common carp is able to cope with these metal levels at least during a one-week exposure.

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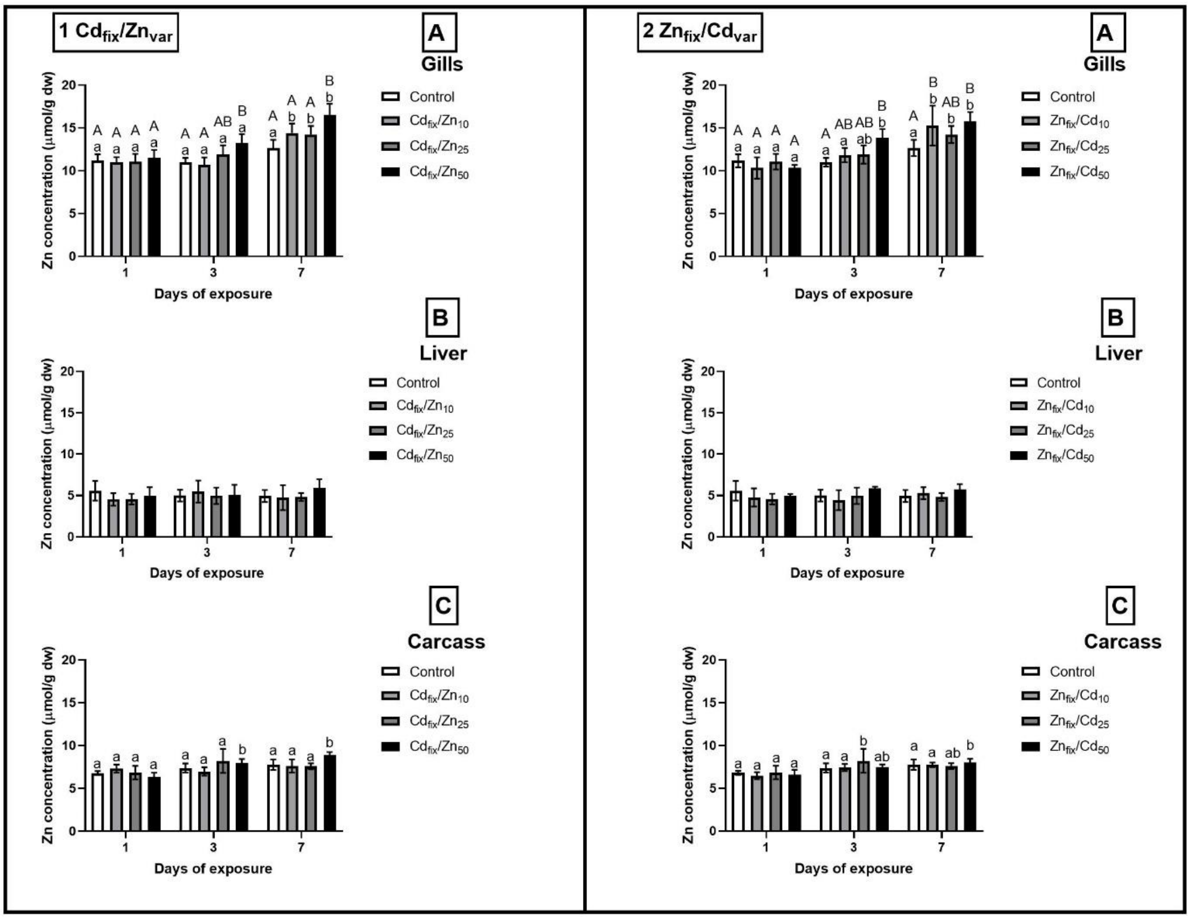


Figure 1: Zn concentration (µmol/g dw) in gills (A), liver (B) and carcass (C) of *Cyprinus carpio* exposed to $Cd_{fix}/Zn_{var}$ (1) or $Zn_{fix}/Cd_{var}$ (2) mixture sampled on day 1, 3 and 7 (mean ± SD, n=5). ). Letters were only added when statistical differences occurred. Lower-case letters denote significant differences (p<0.05) of treatments between sampling days, capital letters indicate significant differences (p<0.05) among treatments within the same sampling day.

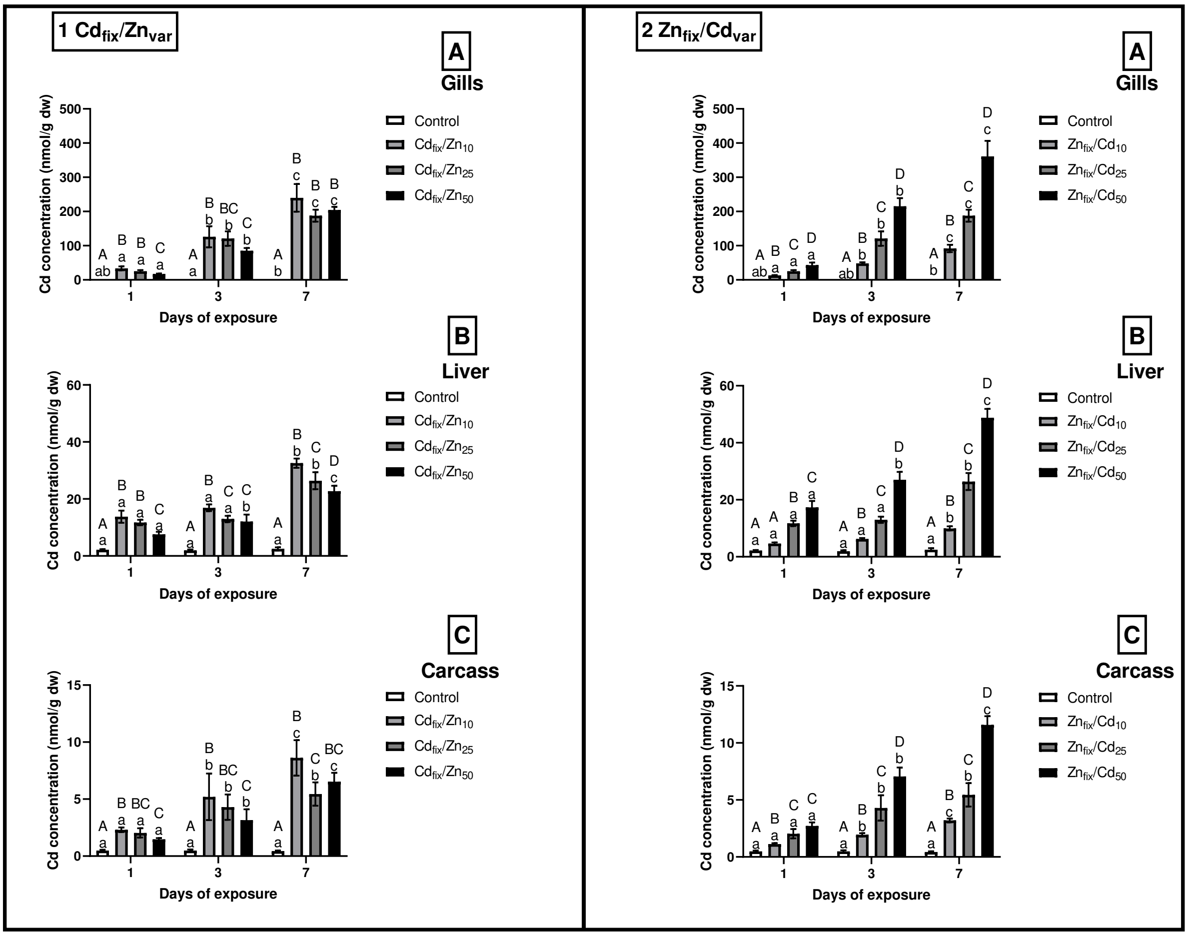


Figure 2: Cd concentration (nmol/g dw) in gills (A), liver (B) and carcass (C) of *Cyprinus carpio* exposed to $Cd_{fix}/Zn_{var}$ (1) or $Zn_{fix}/Cd_{var}$ (2) mixture sampled on day 1, 3 and 7 (mean ± SD, n=5). Letters were only added when statistical differences occurred. Lower-case letters denote significant differences ($p<0.05$) of treatments between sampling days, capital letters indicate significant differences ($p<0.05$) among treatments within the same sampling day.

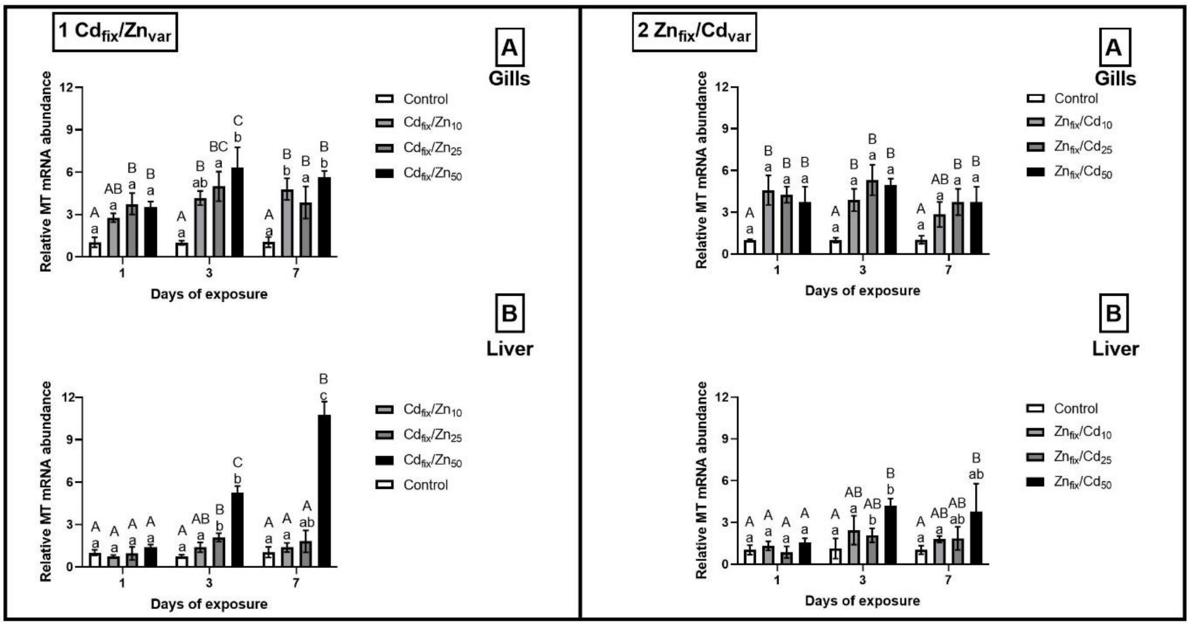


Figure 3: Relative metallothionein (MT) mRNA abundance in gills (A) and liver (B) of *Cyprinus carpio* exposed to $Cd_{fix}/Zn_{var}$ (1) or $Zn_{fix}/Cd_{var}$ (2) mixture sampled on day 1, 3 and 7 (mean ± SD, n=4). Letters were only added when statistical differences occurred. Lower-case letters denote significant differences (p<0.05) of treatments between sampling days, capital letters indicate significant differences (p<0.05) among treatments within the same sampling day.

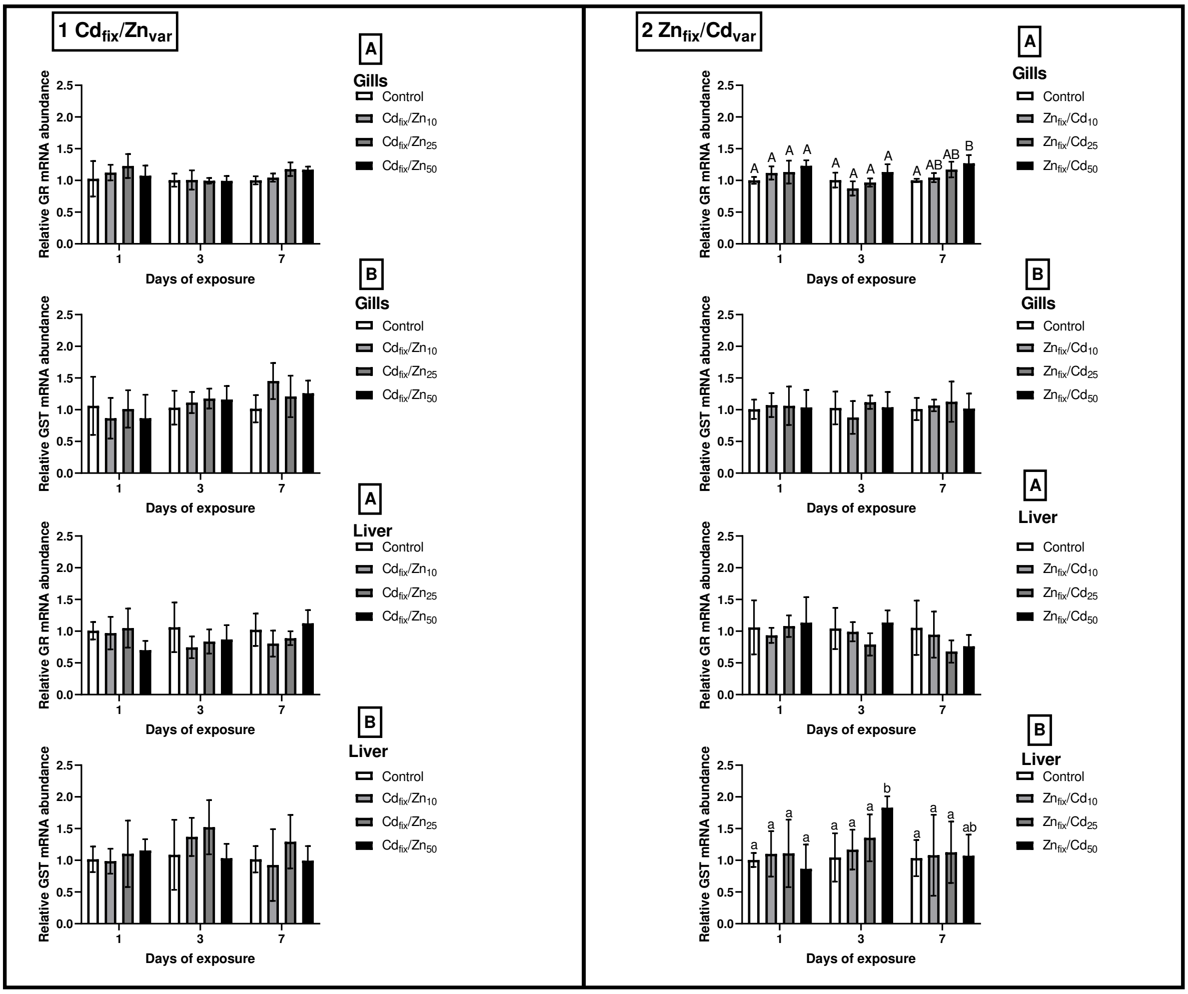


Figure 4: Relative glutathione reductase (GR) (A) and glutathione-S-transferase (GST) (B) mRNA abundance in both gills and liver of *Cyprinus carpio* exposed to $Cd_{fix}/Zn_{var}$ (1) or $Zn_{fix}/Cd_{var}$ (2) mixture sampled on day 1, 3 and 7 (mean ± SD, n=4). Letters were only added when statistical differences occurred. Lower-case letters denote significant differences (p<0.05) of treatments between sampling days, capital letters indicate significant differences (p<0.05) among treatments within the same sampling day.

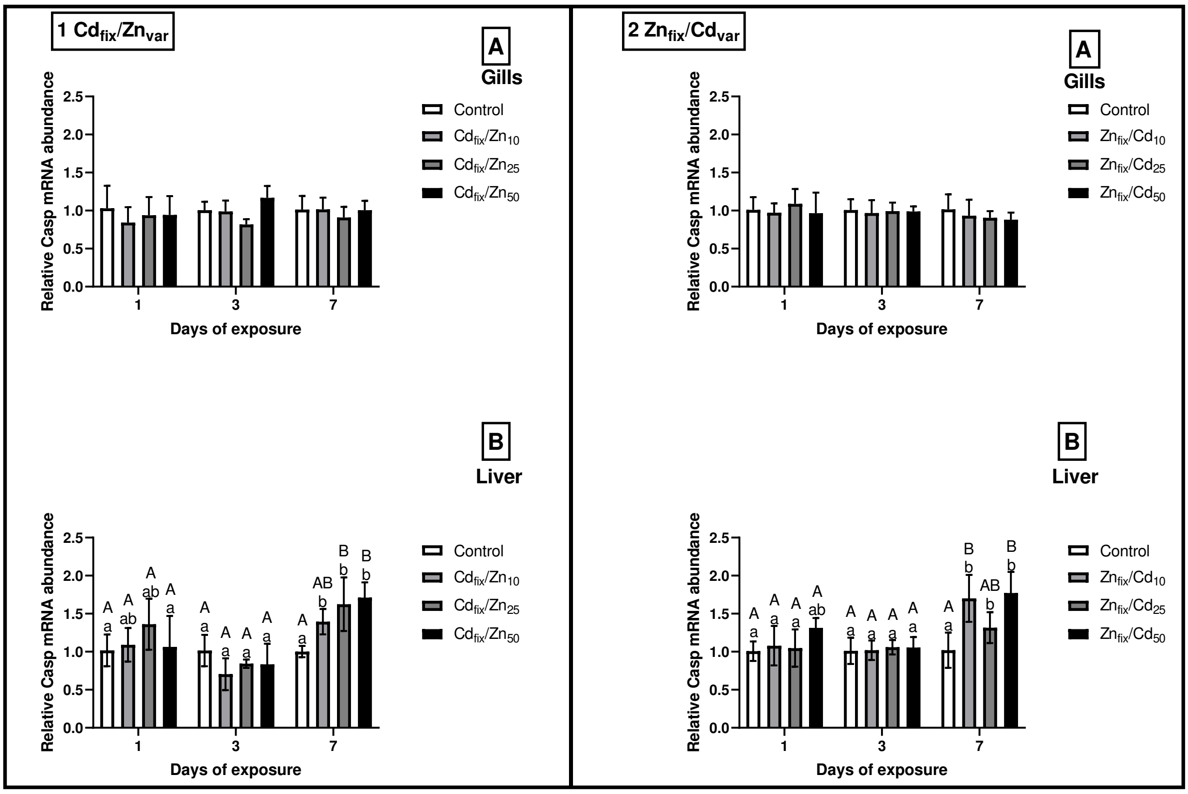


Figure 5: Relative caspase 9 (Casp) mRNA abundance in gills (A) and liver (B) of *Cyprinus carpio* exposed to $Cd_{fix}/Zn_{var}$ (1) or $Zn_{fix}/Cd_{var}$ (2) mixture sampled on day 1, 3 and 7 (mean ± SD, n=4). Letters were only added when statistical differences occurred. Lower-case letters denote significant differences (p<0.05) of treatments between sampling days, capital letters indicate significant differences (p<0.05) among treatments within the same sampling day.

Figure 6



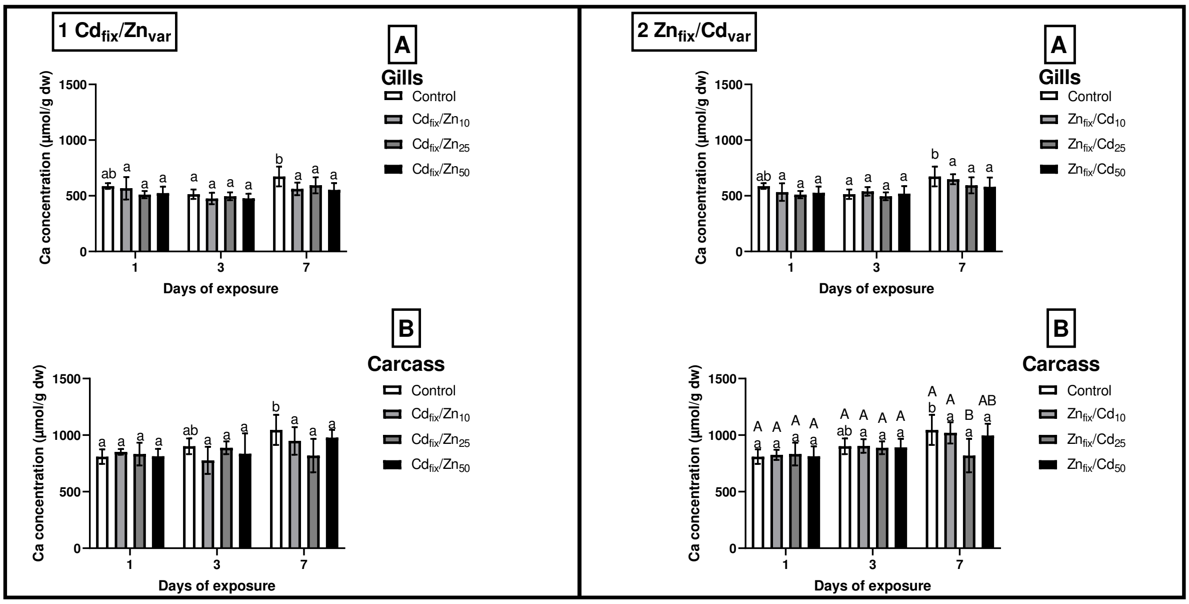


Figure 6: Ca concentration (µmol/g dw) in gills (A) and carcass (B) of *Cyprinus carpio* exposed to $Cd_{fix}/Zn_{var}$ (1) or $Zn_{fix}/Cd_{var}$ (2) mixture sampled on day 1, 3 and 7 (mean ± SD, n=5). Letters were only added when statistical differences occurred. Lower-case letters denote significant differences (p<0.05) of treatments between 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, Investigation, Formal analysis, Writing- Original draft preparation, Writing- Reviewing and Editing**.: Nguyễn Thanh**: Investigation, Formal analysis, Writing - Original draft preparation, Writing- Reviewing and Editing**.: Lieven Bervoets**: Supervision, Funding acquisition**.: Raewyn M. Town**: Conceptualization, Validation**.: Ronny Blust:** Supervision, Funding acquisition**.: Gudrun De Boeck:** Conceptualization, Supervision, Funding acquisition, Writing- Reviewing and Editing formal analysis.