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Reference:
Castaldo Giovanni, Delahaut Vyshal, Slootmaekers Bart, Bervoets Lieven, Town Raewyn M., Blust Ronny, De Boeck Gudrun.- A comparative study on the effects of three different metals (Cu, Zn and Cd) at similar toxicity levels in common carp, Cyprinus carpio
Journal of applied toxicology - ISSN 0260-437X - Hoboken, Wiley, 41:9(2021), p. 1400-1413
Full text (Publisher's DOI): https://doi.org/10.1002/JAT.4131
To cite this reference: https://hdl.handle.net/10067/1744160151162165141

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A comparative study on the effects of three different metals (Cu, Zn and Cd) at similar toxicity levels in common carp, *Cyprinus carpio*.

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Abstract

To improve our understanding of underlying toxic mechanisms, it is important to evaluate differences in effects that a variety of metals exert at concentrations representing the same toxic level to the organism. Therefore the main goal of the present study was to compare the effects of waterborne copper (Cu(II)), zinc (Zn(II)) and cadmium (Cd(II)) on a freshwater fish, the common carp (*Cyprinus carpio*), at concentrations being 0, 25, 50 and 100% of the 96 h LC50 (the concentration which is lethal to 50% of the population in 96 h). All the exposures were performed for a period of one week at 20°C. Our results show a rapid increase in the amount of copper and cadmium accumulated in the gills, while zinc only started to increase by the end of the experiment. All three metal ions increased metallothionein gene expression in both gills and liver. However, clear adverse effects were mainly observed for the Cu exposed group. Cu caused a decrease in Na level in gill tissue, it altered the expression of genes involved in ionoregulation such as Na^+/K^+-ATPase and H^+-ATPase as well as the expression of oxidative stress related genes, such as catalase, glutathione reductase and glutathione-S-transferase. Zinc and cadmium exposure did not alter the ion levels in the gills. In addition no obvious effect of oxidative stress was observed, except for a transient increase in glutathione reductase at the highest cadmium concentration.

Short abstract

Common carp, were exposed to several Cu(II)), Zn(II) and Cd(II) concentrations for a period of one week at 20º C. Our results shows that both Cu and Cd accumulate fast, whereas this was not the case for Zn. Most of the adverse effects were caused by Cu (e.g. on ion balance and ion transporters). All the three metals triggered the response of defensive mechanisms such as metallothionein gene expression (MT).

Keywords: Fish; metal pollution; copper; zinc; cadmium; ionoregulation

1. Introduction:

Metals are among the most common pollutants, they can be found in almost every aquatic ecosystem, and may pose a serious threat due to their persistence, possible toxic effects and their ability to accumulate in the food chain (Pourang 1995, Bervoets et al. 2009).

Generally, the uptake and accumulation of metals in fish is related to the metal concentration and speciation in the environment (Al-Attar 2005, Jezierska and Witeska 2006). Metal exposure can lead to a wide range of toxic effects in fish, such as cytotoxic, hepatotoxic, and histological alterations (Rajeshkumar et al. 2017).

Copper (Cu) is an essential element which is involved in several metabolic processes and it is a component of many proteins (Sevcikova et al. 2011, Pereira et al. 2016). Copper can be taken up via gills through a putative Na^+-channel coupled with the H^+-ATPase, and it can inhibit the activity of the sodium–potassium adenosine triphosphatase (Na^+/K^+-ATPase) activity, reducing sodium (Na) concentrations and altering ion-homeostasis (Wilson and Taylor 1993, De Boeck et al. 2001, Grosell 2011). Therefore the presence of Cu could lead to a competition at the uptake site with a consequent decrease in uptake of Na^+ (Grosell and Wood 2002, Mackenzie et al. 2004, Niyogi et al. 2015).

Zinc (Zn) is an essential element that plays a vital role in the activity of hundreds of enzymes, and is a key player in cellular homeostasis, oxidative stress, aging, and immune response, amongst others (Fırat et al. 2009, Zhao et al. 2014). Zinc uptake starts with the binding of the cation to a negatively charged site situated on the gill surface, followed by internalization (Pagenkopf 1983, Hogstrand and Wood 1996). Zinc can enter the cells via specific transporters or through an epithelial calcium channel (ECaC) causing a competition between calcium (Ca) and Zn at the uptake site (Alsop and Wood 1999, Bury et al. 2003).

Unlike Cu and Zn, cadmium (Cd) is a non-essential metal and can already be toxic at low concentrations (Tunçsoy and Erdem 2014). Similar to Zn^{2+}, Cd^{2+} utilizes the Ca^{2+}-channels as uptake site in the gills and can disrupt Ca^{2+} homeostasis (Alsop and Wood 1999, McGeer et al. 2011).

When metal intake is not balanced with excretion, the accumulation of metal ions might result in toxic effects. These metal ions have the capacity to increase ROS production and induce oxidative stress (Wang et al. 2004, Zheng et al. 2016, Pillet et al. 2019). To cope with oxidative stress, fish have several defensive mechanisms. Antioxidant defences involve superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST) and glutathione reductase (GR) (Livingstone 2001, Sevcikova et al. 2011). The first line of defence for the organism is represented by SOD, which converts the superoxide radical into hydrogen peroxide (H_2O_2), and CAT which reduces H_2O_2 to water (Atli and Canli 2010). The reduced glutathione (GSH) is a tripeptide, which serves as another line of defence against ROS, by acting as a free-radical scavenger in several antioxidant reactions (Peña-Llopis et al. 2003, Pflugmacher 2004). The levels of GSH are regulated by the presence of GR and GST (Dautremepuits et al. 2009, Pillet et al. 2019).

In the present study common carp, *Cyprinus carpio*, was exposed to several concentrations of three different metal ions, being Cu, Zn and Cd. The main aim was to assess the effects of these metal concentrations at a similar toxicity levels in terms of bioaccumulation and related effects. In particular we investigated to which extent, over a one week exposure, Cu, Zn and Cd can

accumulate in gill tissue, alter ion-homeostasis in the gills and, influence the gene expression of enzymes related with ionoregulation (Na^+/K^+-ATPase, H^+-ATPase) as well as the antioxidant system (GST, GR, CAT, SOD) in both the gills and liver of common carp. In addition, the expression of the gene coding for the metal-binding and detoxifying protein metallothionein (MT) was analysed in these two tissues. Metal content in the liver was not analysed, however previous results obtained in our lab showed that these metals can easily accumulate in this tissue (De Boeck et al. 1997, De Smet and Blust 2001).

The nominal concentrations used represent the control, 25%, 50% and 100% of the 96 h LC_{50} (the concentration which is lethal to 50% of the population in 96h) of each metal ion previously determined in our lab. The total nominal metal concentrations were 0.00 µM, 0.19 µM, 0.38 µM and 0.77 µM for Cu, 0.00 µM, 7.50 µM, 15.00 µM and 30.00 µM for Zn, and 0.00 µM, 0.05 µM, 0.10 µM and 0.20 µM for Cd. Although the concentration of total dissolved metal in the exposure medium was different for Cu, Cd, and Zn, the concentrations represent similar toxicity levels as a percentage of the 96 h LC50. The slope of the dose-response curves was similar for all metal ions over the concentration range considered (Delahaut et al. 2020). In fact, the slopes were so steep that all but the 100% 96 h LC_{50} could be considered sublethal. Accordingly, we hypothesised that the severity of the effects at each exposure level would be comparable. However, we know from the work by Delahaut and co-workers (2020) that in fish exposed to Cu and Cd, a fast accumulation in the gills occurred, while for Zn this accumulation was much slower. Therefore, responses in the Zn exposed fish might be delayed. Regarding the electrolyte levels, we expected a loss of total Na for fish exposed to Cu(II), and a loss of total Ca in fish exposed to Cd(II) and Zn(II). Concerning the gene expression for antioxidant enzymes and MT, we expected an increased level for all three metals, as response of the fish to mitigate the possible damage caused by reactive oxygen species.

2. Material and Methods

2.1. Experimental model

Experimental animals, common carp, were obtained from Wageningen University (the Netherlands) and kept in a 1000 L glass aquaria with a photoperiod of 12 h light and 12 h dark at 20°C for several months. Three weeks before the start of each metal exposure fish, average weight 1.81 ± 0.7 g, were transferred to 200 L tanks filled with medium-hard water. Artificial EPA medium-hard water (Weber 1991) was reconstituted using four different salts (VWR Chemicals): $NaHCO_3$ (1.1427 mM), $CaSO_4.2H_2O$ (0.35 mM), $MgSO_4.7H_2O$ (0.5 mM), KCl (0.05 mM) using deionized tap water (Aqualab, VWR International, Leuven, Belgium). The calculated water hardness using nominal concentrations was 84.6 mg/L $CaCO_3$, whereas the water hardness calculated using measured salt concentrations corresponded to 85.6 mg/L $CaCO_3$. Temperature was kept at 20°C and air was provided with aeration stones. Fish were fed with a commercial food pellet (Hikari® Staple™, Klundert, Netherlands) *ad libitum* for the whole acclimation period until they were fasted 2 days prior to the start of the exposure experiment. Experimental methods complied with regulations of the Federation of European Laboratory Animal Science Associations (FELESA) and were approved by the local ethics committee, University of Antwerp (Permit Number: 2015-94 Project 32252).

2.2. Experimental set up

The same experimental design was used for all the single metal exposures. Fish were distributed amongst 6 polypropylene (PP) (5 + 1 as a back-up in case of mortality) double-

walled containers (6 fish in each container), for each single metal concentration used. Each container was filled with 9 L of medium-hard water (conductivity 314 ± 5 µS/cm, pH 7.9 ± 0.1, water hardness 85.6 ppm CaCO3). Metal stock solutions were prepared by adding copper sulphate (VWR Chemicals, CAS number 7758-99-8), cadmium chloride (Merck KGaA®, Darmstadt, Germany, CAS number 34330-64-8) and zinc chloride (Sigma, CAS Number: 7646-85-7) to ultra-pure MilliQ water and added to the exposure water to reach the desired concentrations. The containers was aerated with air stones and to avoid build-up of waste products such as ammonia, fish were not fed and 90% of the water was changed daily. To minimize disturbance to the fish, the perforated inner container was lifted from the outer one for the daily water change. The fish and 1 L of water stayed behind in the inner container, and the remaining 8 L of water in the outer container could easily be replaced after which the inner container was reinserted. Water samples were collected before and after water changes to check metal concentrations. Medium hard water used for the daily change was prepared 24 hours in advance and kept at 20°C. The measured exposure concentrations expressed in µM for Cu, Zn and Cd can be found in SI-table 1. Measurements were performed with a 7700x ICP-MS (Agilent Technologies, Santa Clara, CA, USA). Metal speciation calculated using VMINTEQ can be found in SI-table 2. For speciation analysis, measured metal and salt concentrations in the water were used and only the lowest exposure concentration is shown considering that speciation is independent from the metal concentration.

2.3. Metal accumulation and gill tissue electrolytes.

On day 1, 3 and 7, two fish from each experimental container were euthanized with an overdose of MS222 buffered with sodium bicarbonate (pH 7.0, ethyl 3-aminobenzoate methane-sulfonic acid, 300 mg/L, Acros Organics, Geel, Belgium). Thus, ten fish per treatment were sampled at each sampling day, but as fish were small, samples were pooled per two fish resulting in five samples per treatment and sampling day. The 1st and 4th gill arch of both left and right side were dissected and collected in a 24 h pre-dried, pre-weighed Eppendorf bullet tube. The samples were immediately weighed to obtain the wet weight (ww) and frozen in liquid nitrogen until further analysis. Samples and reference material (SRM-2976, Mussel tissue, National Institute of Standards and Technology, Gaithersburg, MD, USA) were dried for at least 48 h at 60°C and allowed to cool in a desiccator for more than two hours before recording the dry weight (dw) with a precision scale (Sartorius SE2, Ultra Microbalance). The digestion process was performed according to Reynders et al. (2006) and Blust et al. (1988). Briefly the process started with a digestion of the sample at room temperature for 12 h with trace metal grade HNO_3 (69%) (Seastar Chemicals, Canada) followed by a microwave digestion of three steps at 100 W for three minutes and three steps at 180 W for three minutes. Subsequently, H_2O_2 (29%) (Seastar Chemicals) was added followed by a fourth microwave digestion step at 300 W for two minutes. At the end of the digestion process, 1 ml of ultrapure Milli-Q (MQ), was added to the samples. After that the samples were diluted to a final acid concentration of ~ 2% with MQ water and metal content was analysed using a 7700x ICP-MS (Agilent Technologies, Santa Clara, CA, USA) while electrolyte content was analysed using an iCAP 6300 Duo (Thermo Scientific, Waltham, MA, USA).

2.4. RNA extraction and real time PCR

The 2nd and the 3rd gill arch of five individual fish and five pooled liver samples were used for RNA extraction and gene expression. Total RNA was extracted using Trizol (Invitrogen, Merelbeke, Belgium) following the manufacturer's instructions. The RNA quantity and purity

was evaluated with Nano-Drop spectrophotometry (NanoDrop Technologies, Wilmington, DE) and the integrity with a 1% agarose gel with ethidium bromide (500 µg/mL). DNase treatment was performed using the commercial kit DNase I, RNase free kit 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). Four samples, for each treatment and sampling day, were selected according to the OD260/OD280 nm absorption ratio (higher than 1.8) and used for qPCR. Real-time PCR was performed using a Mx3005P QPCR System (Agilent Technologies, Belgium). Real-time PCR analyses were performed in duplicate in a final 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 Mx3005P QPCR system. Oligonucleotides primers for Na^+/K^+-ATPase were designed using NCBI resources Primer blast and synthesized as highly purified salt-free "OliGold" primers by Eurogentec (Eurogentec, Seraing, Belgium). Primer sequences and primer efficiency are given in SI-Table 3. The efficiency was determined based on the slope of the standard curve, using a serial dilution of cDNA.

2.5. Statistical analysis

All data are presented as mean values ± standard deviation (SD). For the statistical analysis, normality of the data was tested with the Shapiro-Wilk test. If the data were not normally distributed data would be log transformed. For comparisons between different experimental groups a two-way analysis of variance (ANOVA) was performed followed by a Tukey test using GraphPad Prism version 7.04 for Windows, GraphPad Software, La Jolla California USA. The same software was used for curve fitting the metal accumulation (linear, Michaelis-Menten) and sodium loss (two-phase decay) as a function of time and exposure concentration.

3. Results

Measured exposure concentrations (SI-table 1) were on average almost 20% lower than the intended nominal exposure concentrations. This difference was most pronounced at the lowest exposure concentration (25% 96 h LC50) with approximately 30% lower values, which we ascribe to adsorption onto the PP containers used for the exposures. It was much less pronounced at the higher exposure levels (50 and 100% 96 h LC50) which showed on average values which were 10% below the intended exposure level.

3.1. Metal accumulation in the gills

Copper (Fig. 1A) already showed a significant accumulation after one day of exposure. All the exposed groups showed a significant increase compared to the control fish at all the analysed days. However, it was only after seven days that we could observe differences between the different exposure concentrations. At day 7 the copper concentration was almost two times higher in the group exposed to 0.69 µM compared to the 0.14 µM exposed group. Moreover, the accumulation in the groups exposed to 0.36 µM and 0.69 µM was higher at day 7 compared to the same groups at day 1 and 3. From day 1 onwards, copper accumulation was approximately linear in time within each exposure condition (see supplementary information, SI-Fig 1A), but when looking at the different exposure levels per time point (see SI-Fig 1B) there was an apparent saturation at the highest concentrations during the first days, which disappeared at day 7.

Regarding Zn (Fig. 1B), no differences were found between treatment and control during the first three days of exposure. However at day 7, the Zn concentration was significantly elevated in all three exposed groups and saturation of the accumulation seemed to occur at the highest exposure level (see SI-Fig 2B). Zinc accumulation seemed to be linear in time in the exposed fish (see SI-Fig 2A), despite the lack of significance before day 7, and Zn levels in control fish showed a decreasing trend.

For Cd accumulation (Fig. 1C), a significant increase in the treatments compared to the control, occurred from the first day of exposure onwards. Moreover the concentration in the group exposed to 0.03 µM is significantly lower compared to the other two treatments. By the end of the experiment dose-dependent differences between exposed groups were more prominent. Furthermore at day 7 the metal accumulation was higher in all the treatments compared with the start of the experiment. The accumulation at day 7 was higher in the groups exposed to 0.08 µM and 0.18 µM compared to the same groups at day 1 and 3. Cadmium accumulation was obvious after 1 day of exposure and continued until day 7 without reaching a steady-state, both through time and over the different exposure levels (see SI-Fig 3B and 3C). More information on the metal accumulation is given in the SI-tables 4, 5, 6 and 7.

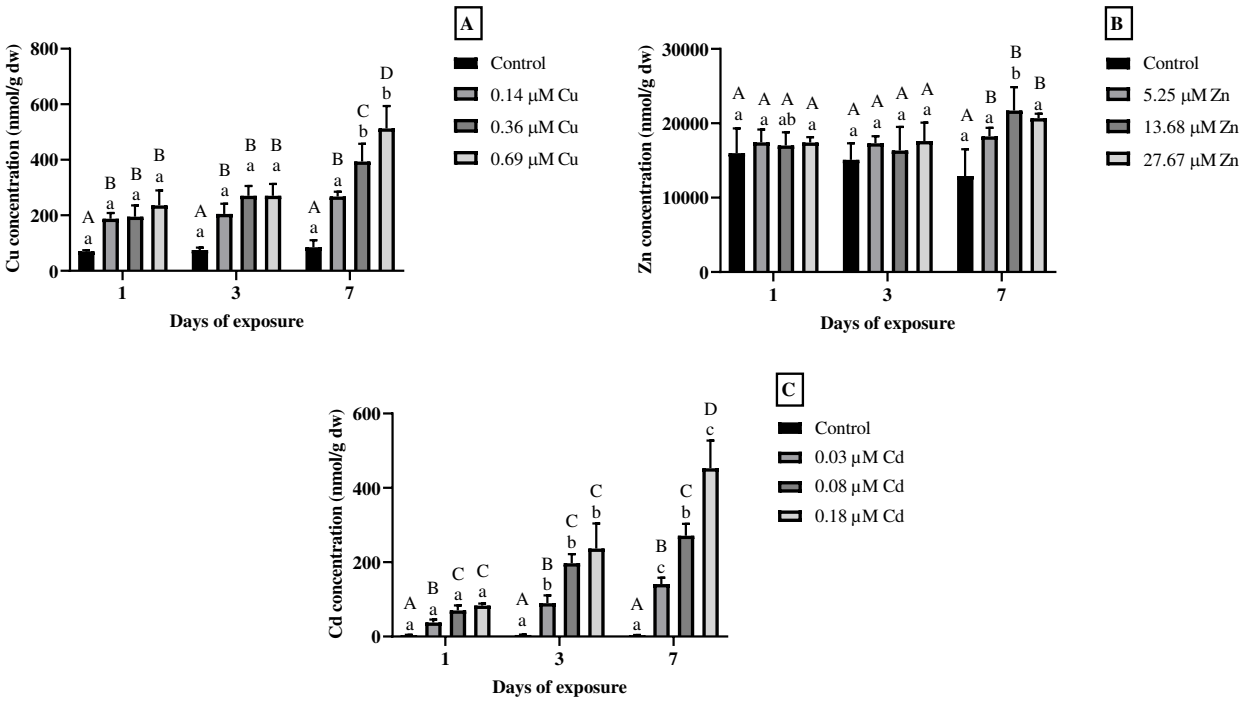

Figure 1: (A) Cu, (B) Zn, (C) Cd accumulation in the gills (nmol/g dw) of *Cyprinus carpio* exposed to different metals concentrations for 1, 3 and 7 days. Capital letters indicate significant differences between treatments during the same sampling day ($p < 0.05$). Lowercase letters indicate significant differences between the same treatment at different sampling days ($p < 0.05$).

3.2. Defensive mechanisms

3.2.1. Metallothionein gene expression

3.2.1.1. Gills

MT gene expression in gills of fish exposed to Cu increased quickly (Fig 2.A). After one day, gene expression in all exposed groups was already significantly elevated compared to the control. This elevation was dose-dependent as fish exposed to 0.69 µM showed a significantly higher elevation in the expression compared to fish exposed to 0.14 µM and 0.36 µM. At day 3 the expression in the group exposed to 0.14 µM had returned to control levels, and by the end of the experiment only the group exposed to 0.69 µM showed an elevated gene expression rate compared to the control. Regarding MT mRNA expression in fish exposed to Zn (Fig 2.B) we observed a stimulation for all the exposed groups compared to the control from day 1 onwards, with the exception of the lowest exposure level which returned to control levels at day 7. This increase occurred in a dose-dependent way from day 3 onwards. For fish exposed to Cd (Fig 2.C), the MT gene was overexpressed in all the treatments compared to the control during the whole experiment, but the dose-dependent effect was much less clear.

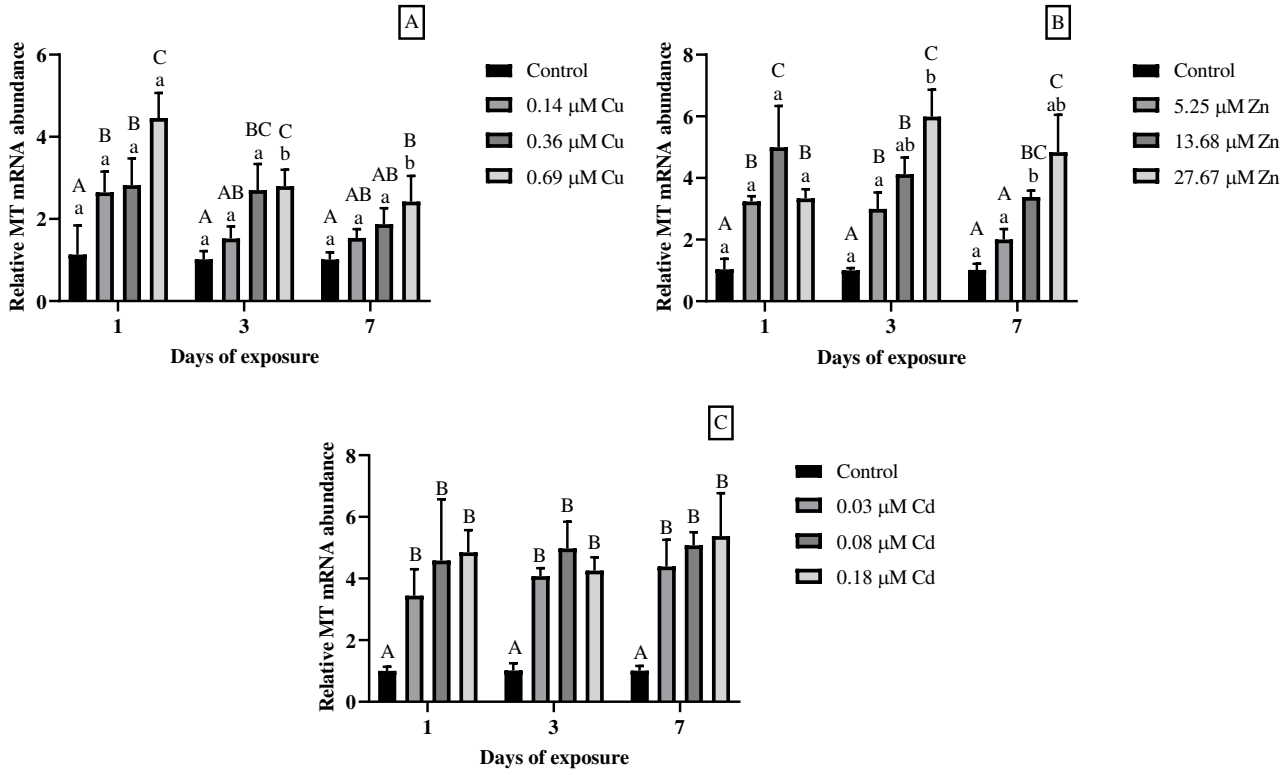

Figure 2: Relative MT mRNA abundance in *Cyprinus carpio* gills of fish exposed to different concentrations of (A) Cu, (B) Zn and (C) Cd, for 1, 3 and 7 days (mean ± SD) (N=4). Capital letters indicate significant differences between treatments during the same sampling day ($p < 0.05$). Lowercase letters indicate significant differences between the same treatment at different sampling days ($p < 0.05$).

3.2.1.2. Liver

MT gene expression in the liver of fish exposed to Cu (Fig 3.A) showed a significant but transient increase in all treatments compared to the control at day 3, after which the expression

went back to control levels by day 7. Regarding fish exposed to Zn (Fig 3.B) we observed an increased expression in fish exposed to 27.67 µM both at day 3 and day 7. In fish exposed to Cd (Fig 3.C) our results showed a transient increased gene expression after one day of exposure for the two highest exposure groups of 0.08 µM and 0.18 µM.

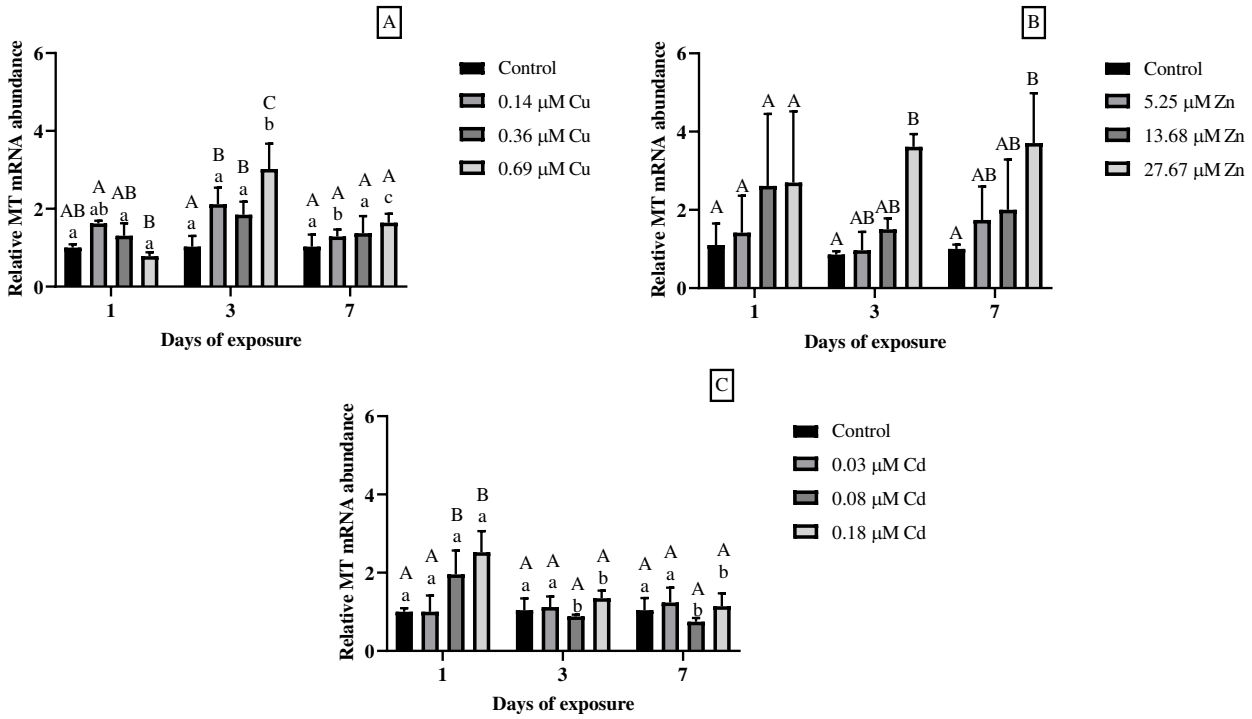

Figure 3*:* Relative MT mRNA abundance in *Cyprinus carpio* liver of fish exposed to different concentrations of (A) Cu, (B) Zn and (C) Cd, for 1, 3 and 7 days (mean ± SD) (N=4). Capital letters indicate significant differences between treatments during the same sampling day ($p < 0.05$). Lowercase letters indicate significant differences between the same treatment at different sampling days ($p < 0.05$).

3.2.2. Antioxidant related gene expression

In fish exposed to Cu, CAT gene expression in the gills (Fig 4.A) was reduced in fish exposed to 0.69 µM after one day of exposure, followed by a recovery at day 3 and 7. In the liver (Fig 4.B) the pattern was similar with a reduced expression at day one for the group exposed to 0.69 µM followed by recovery. The GST mRNA expression in the gills (Fig 4.A) showed a reduction for the group exposed to the highest concentration at day 1 with only a partial recovery after 3 days. However by the end of the experiment, full recovery had occurred and no more significant differences were observed between control and exposed groups. In the liver (Fig 4.B) the GST gene expression was reduced in the group exposed to 0.69 µM at day 1; and at day 3 we also observed a similar reduction in GST gene expression in fish exposed to 0.14 and 0.36 µM. However at day 7 we observed a recovery to control levels for all the treatments. GR gene expression in the gills (Fig 4.A) was increased in groups exposed to 0.36 µM and 0.69 µM Cu after one day of exposure. Overall, the gene remained overexpressed until the end of the experiment. In the liver (Fig 4.B) no differences were seen between control and treatments. SOD mRNA expression in the gills (Fig 4.A) showed a decreasing trend at day one in the treatments compared to the control, with a significant decrease in the group exposed to 0.69 µM. However from day 3 onwards a recovery was observed. More details about mRNA gene expression during Cu exposure can be found in SI-table 8.

For fish exposed to Cd, we did not find any differences in mRNA expression of the genes related to oxidative stress between control and treatment in either of the tissues, except for GR in the gills (see SI-table 9) where we observed a significant increase in the treatment at 0.18 µM on day 3.

Also the gene expression of antioxidant enzymes in fish exposed to Zn showed no differences between control and treatments for either tissue (see SI-table 10).

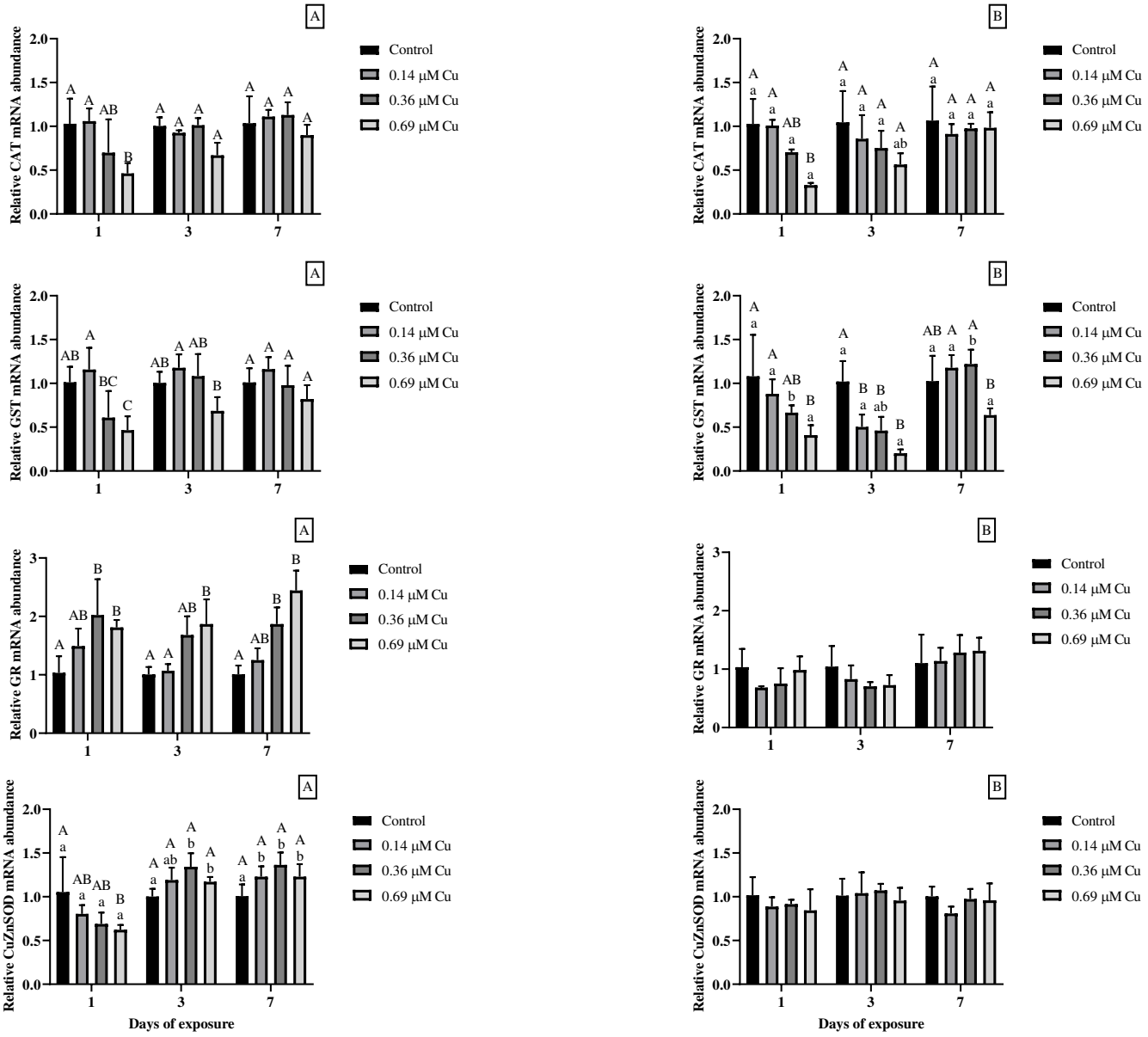

Figure 4: Relative CAT, GST, GR and CuZnSOD mRNA abundance in *Cyprinus carpio* (A) gills and (B) liver exposed to different Cu concentrations for 1, 3 and 7 days (mean ± SD) (N=4). Capital letters indicate significant differences between treatments during the same sampling day ($p < 0.05$). Lowercase letters indicate significant differences between the same treatment at different sampling days ($p < 0.05$).

3.3. Tissue electrolyte levels

Sodium content (Fig. 5) in the gills of fish exposed to 0.69 µM Cu already showed a significant decrease compared to the control group and the group exposed to 0.14 µM after one day. At

day 3 and 7 all the exposed groups showed a significant decrease in Na content compared to the control. At day 7 the decrease in Na was dose-dependent and more accentuated in fish exposed to 0.69 µM in comparison to fish exposed at 0.14 µM. The rate of Na loss for the lowest concentration increased from day 1 to day 3, after which it slowed down at day 7 shown by the two-phase decay in Na concentrations expressed over time (See SI-Fig 4.A), while at the highest exposure concentration we observed the highest loss rates at day 1 followed by a more linear Na loss thereafter. As a consequence, the clear linear dose-dependent Na loss at day 1, with slower Na loss at lower Cu exposure levels and fast Na loss at the highest exposure level (See SI-Fig 4B), levels off through time into a plateau and Na loss at the two higher Cu exposure levels were very similar.

Regarding the magnesium (Mg) levels in the gills of Cu exposed fish, our results showed a transient increase compared to the control in fish exposed to the highest concentration at day 3 (see SI-table 4). For Ca and potassium (K) in Cu exposed fish, no differences were found between control and treatments (see SI-table 4).

For fish exposed to Zn and Cd no differences in Ca levels, or other electrolytes, were observed, (see SI-table 5 and 6).

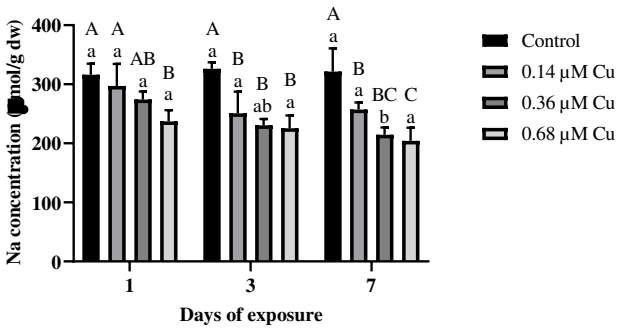

Figure 5: Na levels (µmol/g dw) in the gills of *Cyprinus carpio* exposed to different concentrations of Cu for 1, 3 and 7 days (mean ± SD) (N=5). Capital letters indicate significant differences between treatments during the same sampling day ($p < 0.05$). Lowercase letters indicate significant differences between the same treatment at different sampling days ($p < 0.05$).

3.4. Ionoregulation related gene expression

H^+-ATPase mRNA expression increased significantly in a dose dependent manner in all the treatments after one day of exposure. At day 3 only the gene expression of the group exposed to 0.69 µM remained significantly elevated compared to the control and by the end of the experiment the expression had returned to baseline levels (Fig. 6). In contrast, Na^+/K^+-ATPase mRNA expression only showed an increase at day 1 in fish exposed to 0.69 µM, followed by a decrease in all the treatments compared to the control at day 3. Subsequently, we observed a recovery at day 7 for all the treatments (Fig. 6).

In fish exposed to Zn and Cd, no differences were found between control and exposed groups in H^+-ATPase and Na^+/K^+-ATPase gene expression (see SI-table 9 and 10).

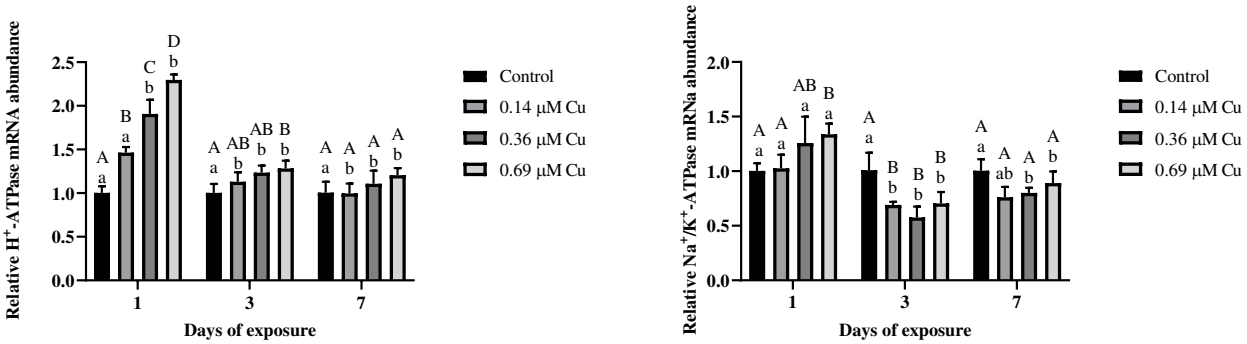

Figure 6: Relative H^+-ATPase and Na^+/K^+-ATPase mRNA abundance in *Cyprinus carpio* gills exposed to different Cu concentrations for 1, 3 and 7 days (mean ± SD) (N=4). Capital letters indicate significant differences between treatments during the same sampling day ($p < 0.05$). Lowercase letters indicate significant differences between the same treatment at different sampling days ($p < 0.05$).

4. Discussion

The main aim of the present work was to compare different single metal exposures at similar toxic levels to understand how each metal accumulates, impacts ion-homeostasis and induces protective mechanisms over a one week exposure. As mentioned above, measured exposure concentrations were lower than the intended nominal exposure concentrations probably due to adsorption to the exposure tanks and actually approximated 17, 45 and 90% of the 96h-LC50. However, as the percentage adsorption of each metal at each exposure level was similar, the comparison between the different metals remains valid. It did have the important consequence that a reduced mortality only occurred in the Cu exposure scenario, in which only one fish died at the highest metal concentration, confirming the steep mortality curves previously observed (Delahaut et al. 2020).

4.1. Cu, Zn and Cd accumulation.

Comparing accumulation of the single metals at similar toxicity concentrations made it clear that Cu and Cd accumulated faster to significantly elevated levels when compared to Zn. This is despite the fact that total Zn levels and accumulated concentrations were higher, partly reflecting the higher exposure levels. Therefore, delayed toxicity could have been expected in Zn exposed fish. However, Zn accumulation did increase at a constant rate throughout the experiment indicating that it was taken up from the start of the exposure onwards. When comparing Cu and Cd exposed fish, net accumulated metal and accumulation rates in the gills at the first day of exposure were higher in Cu exposed fish compared to Cd exposed fish, but by day 3 and 7 the accumulation rates of the two metals were similar. When comparing the effects of the metal concentrations on toxicity mechanisms, our results show that Cu is the metal which caused most negative effects, while this was not the case for Cd and Zn. Even though all the metals seem to stimulate the induction of MT gene as defence mechanism, Cu also altered Na homeostasis in the analysed tissue, whereas Cd and Zn at comparable toxicity levels did not show any effect on electrolyte content.

Our results showed a clear time dependent Cu accumulation which was very fast at the start, independent of the exposure concentration. By the end of the experiment the Cu content in the tissue increased proportionally to the exposure condition and differences between the treatments were observed. Such a fast Cu accumulation at the onset of the exposure was

expected as the gills are in direct contact with the water and are the primary uptake site of the animal under waterborne exposure (Grosell et al. 1997, Niyogi and Wood 2004). This observation is no surprise because the conditional equilibrium constant for metal-gill binding sites is high (log *K* 7.4 (dm^3 mol^{-1}) (Playle 2004), leading to a fast and strong interaction with the gill's binding and uptake sites. After this first phase, and similar to what was found in gibel carp (De Boeck et al. 2003), Cu accumulation showed a linear trend in time. Gills are just a temporary target organ for metal accumulation, because the metals are subsequently transported via the blood stream to the liver and kidney for excretion via the hepatobiliary system (Grosell 2011, Kondera et al. 2014). This seems confirmed in our experiment, although accumulation rates slowed down towards day 7. It suggests that transfer to the blood, followed by excretion through the renal and hepatobiliary system was slowly adapting to counteract the increased influx of Cu towards the end of the exposure, and that at least at the 25 and 50% 96 h LC50 levels, carp are able to either reduce Cu influx or stimulate Cu transfer from the gills to other organs. However, despite the reduced accumulation rate, gill Cu concentrations at day 7 still increased significantly compared to the previous days. At this time accumulation between treatments had clearly become dose dependent. At the 100% 96 h LC50 level, physical gill damage might have allowed the Cu to enter the gills more easily from the start (De Boeck et al. 2004); previous work has shown that gill damage was present over the entire period in a one week exposure of common carp to 1 µM Cu (De Boeck et al. 2007).

Cadmium is considered to be a non-essential metal toxic for aquatic organisms (Matsuo et al. 2005). Cadmium showed a clear time and dose-dependent accumulation, which is also reflected in the accumulation rates. Similar to Cu, the Cd accumulation rate for the lowest exposure concentration is higher during the first day of exposure and then slows down towards day 7, while for fish exposed to 0.08 µM and 0.18 µM the accumulation rates remain at a high and more constant level for the whole week. This corresponds well with the very high affinity Cd has for gill binding sites, with a conditional log*K* of 8.6 (dm^3 mol^{-1}) (Playle 2004) it binds to gills about 16 times stronger than Cu and 1000 times stronger than Zn under equal exposure conditions. We estimated Cd accumulation at 3 h from results obtained by Van Ginneken et al. (1999) for common carp and these corresponded to 18, 42 and 77 nmol/g dw which is close to our values at day 1. Therefore we can assume that also for Cd, fish started to accumulate metal ions within the first hours of exposure. A similar Cd accumulation has been observed in different species such as in the gills of rainbow trout (Hollis et al. 1999), in the liver of *Trematomus bernacchii* (Illuminati et al. 2010), gills liver and kidney of sea bream (*Sparus aurata*) (Isani et al. 2009), in the gills of juvenile olive flounder (*Paralichthys olivaceus*) (Kim et al. 2004) and in common carp gills (De Smet and Blust 2001). Furthermore, a concentration-dependent increase of Cd was evident in a field study on caged common carp, which accumulated more Cd in the location with the higher metal concentrations (Reynders et al. 2008).

No concentration dependent gill Zn accumulation could be observed only by the end of the exposure period. Therefore, exposure time, rather than the exposure concentration, seems to be more important for Zn accumulation. A similar delay in significant accumulation was also observed in other studies, for instance, McGeer et al. (2000) found that branchial Zn accumulation in rainbow trout only started after 10 days of exposure. To explain this delay in the accumulation we have to take into account that Zn is one of the most abundant ions in the body, therefore its accumulation and excretion is highly regulated in the organism (Zhao et al.

2014). For example in a field study done with gibel carp, branchial Zn concentration was in the same range between the different sampling sites in a Zn concentration gradient in the water (Van Campenhout et al. 2010). Again, using data from an earlier study (Van Ginneken et al. 1999), an estimated Zn accumulation at 3 hr of 832, 1095 and 1216 nmol/g dw can be calculated under our nominal waterborne metal levels, which is close to the values we found at day 1 and 3. This suggests that Zn uptake actually starts very rapidly, despite the fact that under the same conditions the conditional equilibrium constant for gill binding sites is much lower compared to that for Cu: Zn binds about 63 times less strongly to the gill ($\log K$ 5.6 (dm^3 mol^{-1})) (Playle 2004). Therefore in light of this observations, we can suggest that this delay in significant accumulation was due to the ability of the fish to regulate Zn uptake, and to the naturally high background zinc content and not to the fact that it was not taken up. The latter is supported by the fact that, despite the delay in a significant accumulation, metal content increased linearly from the start of the exposure onwards. Furthermore we can conclude that Zn uptake processes were efficiently compensated by depuration and regulatory processes.

4.2. Defensive mechanisms

Metallothionein are involved in the transport and storage of metals and provide a protective role against their toxic effects by reducing the concentration of free metal ions (Hamilton and Mehrle 1986, De Boeck et al. 2003). Previous studies showed that the affinity of MTs for different metal ions follows the order: $Hg^{2+} > Cu^{+}$, Ag^{+}, $Bi^{3+} \gg Cd^{2+} > Pb^{2+} > Zn^{2+} > Co^{2+}$ (Vašák 1991). In our study all the metal ions tested showed the ability to induce the expression of the MT gene both in the gills and in the liver. In gills, MT mRNA induction occurred within the first day and was both long-lasting and non-dose dependent for Cd, and more subtly dose-dependent for Cu and Zn where the induction of MT gene expression was transient at the lower exposure concentrations. In the present study an increased gene expression for Cu and Cd was expected considering the fast accumulation of these metals in the tissue, while for Zn a MT gene expression induction was only expected in concomitance with the Zn increase. However, as mentioned above, Zn influx and accumulation was linear from the start, so new Zn did enter the cells from the start of the exposure onwards (as evidenced by the data of Van Ginneken et al., 1999). Probably the transient increased MT gene expression can be explained by the role of Zn in the activation of metal regulated transcription factors which starts the MT gene expression process (Roesijadi 1996, Sevcikova et al. 2011). As in our study, increased MT gene expression was found during Cu exposure in the liver of Javanese Medaka (*Oryzias javanicus*) exposed to 10, and 100 ppb of Cu (Woo et al. 2006), and in both the gills and the liver of zebrafish exposed to Cu (Craig et al. 2009). We found a dose dependent induction, following the dose dependent Cu accumulation. It was no surprise that the fast increase in gill Cd levels also caused an immediate induction of MT gene expression. Similar responses to Cd have been found in goldfish, coho salmon (Choi et al. 2007, Espinoza et al. 2012) and even in swim-up and early life stages of rainbow trout and white sturgeon (*Acipenser transmontanus*) (Shekh et al. 2019). In contrast to the Cu and Zn exposure however, MT mRNA induction seemed to be an on-off event and did not follow the dose dependent Cd accumulation seen in the gills.

In general the MT gene was continuously increased in the gills of the exposed groups, whereas that was not always the case in the liver. The transient induction of MT gene expression observed in the liver, occurred mainly at the highest exposure concentrations and, except for

Cd, was delayed to day 3 or 7. As said above MTs have a role in essential metal homeostasis and background levels of the protein, which plays a detoxifying role, are always present in liver (De Boeck et al. 2003). Several authors showed that MT are present in control fish, with higher levels in the liver compared to the gills (De Smet et al. 2001, Hollis et al. 2001, Chowdhury et al. 2005, Hashemi et al. 2008). When fish are exposed to metal ions, extra thionein synthesis is only induced when needed (Hamilton and Mehrle 1986).

Reactive oxygen species are produced naturally during metabolism, but due to antioxidant enzymes and vitamin D they are normally prevented from causing toxic effects (Hansen et al. 2006). Although metal ions can increase the ROS production (Rajeshkumar et al. 2017), defensive mechanism such as SOD, CAT, GST and GR are involved in ROS removal (Hansen et al. 2006, Pillet et al. 2019). In the present study we investigated changes in the gene expression of SOD, CAT, GST and GR in the gills as well as in the liver. In the Cu exposed group the general gene expression trend in the gills for CuZnSOD, CAT and GST showed a reduction in the 0.69 µM exposed group after one day of exposure with a subsequent recovery, while GR mRNA increased in fish exposed to 0.36 µM and 0.69 µM from day 1 onwards. A similar pattern was observed in the liver. Inhibitory responses of antioxidant enzymes can be caused both by Cu - that binds the -SH group of the enzyme - or by the excess of ROS (Sanchez et al. 2005, Atli and Canli 2007, 2010) and are indicative of contamination (Hansen et al. 2007, Díaz-de-Alba et al. 2017). An inhibition of SOD activity by Cu was reported in three spine stickleback (*Gasterosteus aculeatus*) (Sanchez et al. 2005). In previous studies either an increase or a decrease of CAT activity was observed according to metal concentrations and tissues studied (Jia et al. 2011, Díaz-de-Alba et al. 2017, Pan et al. 2018). Regarding GST, an enzyme which conjugates GSH with electrophilic and other xenobiotics, a decline was recorded in different organisms exposed to various metal ions, such as Nile tilapia (*Oreochromis niloticus*) (Atli and Canli 2010), the freshwater snail *Lymnea luteola L.* (Ali and Ali 2015) and in common carp exposed to different concentrations of Cu (Dautremepuits et al. 2004, Pillet et al. 2019). For GR, an enzyme involved in the restoration of GSH, an upregulation of the gene was recorded in several fish exposed to Cu such as sea bream (Minghetti et al. 2008). According to Eyckmans et al. (2011), common carp primarily rely on the GSH as first line of defence and the binding of metal ions with this antioxidant can lead to the depletion of reduced GSH. Thus GR is needed to restore and maintain the GSSG/GSH ratio. In the present study the levels of GSH/GSSG were not measured, however the results obtained let us assume that on one hand a depletion of GSH occurred, while on the other hand any use of GSH in carp was counteracted efficiently by the GR. Moreover the upregulation of MT and GR together with the recovery of GST, CAT and CuZnSOD showed that common carp were affected by metal ion exposure but quickly adapted to the adverse situations.

4.3. Disturbance in ionoregulation

In the present experiment we expected an initial reduction of Na for fish exposed to Cu with an increased gene expression for H^+-ATPase and Na^+/K^+-ATPase to counteract this loss, while for fish exposed to Zn and Cd we expected a decreased level of Ca.

Our data show a Na decrease within the first day for fish exposed to 0.69 µM of Cu, and after 3 days this decrease was clear also in the remaining treatments. A similar Na decrease in the gills of Nile tilapia exposed to different concentration of Cu has been reported (Atli and Canli 2011). Moreover, the Na loss due to Cu exposure was already recorded in common carp, in the neotropical *Prochilodus scrofa* and in rainbow trout (De Boeck et al. 2001, Cerqueira and

Fernandes 2002, Grosell and Wood 2002). Often Na loss is linked with the onset of mortality in fish. For example in rainbow trout and yellow perch mortality occurred with a Na body loss between 30 to 40% (Taylor et al. 2003), while for gibel carp the onset of mortality is a reduction with ~ 45% Na (De Boeck et al. 2010). Thus the low mortality in the present experiment, in addition with the lower intended metal concentrations in the media and the LC50 curves shape (Delahaut et al. 2020) could also be linked with the relatively low Na loss in the gills.

We analysed the gene expression of H^+-ATPase and Na^+/K^+-ATPase in order to better understand the fish's response to the Na loss. Sodium can enter into the gills in different ways: through a putative sodium channel energized by an electrical gradient created by H^+-ATPase, through a Na^+/H^+ exchanger or through a Na^+/Cl^- cotransporter (McCormick 2001, Grosell 2011, Kumai and Perry 2012). Furthermore the Na^+/K^+-ATPase present in the branchial cells is associated with Na^+ transport and together with the Na^+/H^+ exchanger creates an electrochemical gradient for Na^+ uptake (Lin and Randall 1993, McCormick 2001). Our results show the ability of Cu to affect Na content togheter with the expression of H^+-ATPase and Na^+/K^+-ATPase. The ability of Cu to inhibit the Na^+/K^+-ATPase activity has already been demonstrated by several authors in several species such as Mozambique tilapia, common carp and rainbow trout (Li et al. 1998, De Boeck et al. 2001, Chowdhury et al. 2005, Hashemi et al. 2008). Moreover an inhibition of the enzyme was also reported in zebrafish by Craig et al. (2009) who reported an increase in the gene expression of the enzyme together with inhibition of the activity. Therefore we expected an increased gene expression for the Na^+/K^+-ATPase to cope with the Na loss. However our results showed a significant increase between control and treatment only in the highest Cu treatment at day 1. After that a downregulation of the gene was observed at day 3, followed by a recovery at day 7 for all the Cu concentrations. This trend is in line with the results obtained for the Na content which dropped at day 3 in all the Cu exposed groups, possibly as a consequence of this reduced Na^+/K^+-ATPase gene expression. The subsequent gene recovery at day 7 suggests that the organism is trying to cope with this situation, which seemed successful considering that the electrolyte levels in the different exposed groups remained stable with no further loss between day 3 and day 7. For the H^+-ATPase we observe a dose dependent gene upregulation after one day of Cu exposure. One might assume that this represents an attempt by the fish to enhance the Na uptake, through the release of H^+ in the extracellular medium to generate an electrochemical gradient. This in turn would favour the sodium entry through the putative apical sodium channel. This increase of the H^+-ATPase could also explain why the Na decrease only occurred after 3 days of exposure for fish exposed to 0.14 µM and 0.36 µM of Cu.

Even though Cd and Zn are known to interfere with Ca homeostasis in fish, such as rainbow trout and in galaxiid fish (McRae et al. 2016, Shekh et al. 2018), in our experiment Ca levels were not affected by these metals. We hypothesize that this could be due to the short exposure period combined with the relatively high background Ca levels, therefore a longer exposure at lower water hardness is needed to validate this hypothesis.

5. Conclusion

The present study shows the ability of common carp to cope and adapt in adverse situations, even with significant amounts of metal ions present. We confirmed that Cu and Cd accumulated quite fast, and Zn accumulation was delayed. According to our results, defence mechanisms are upregulated for all the metals, but to a different extent. Especially MT gene expression was

induced quickly for all exposures, indicating that detoxification of the new incoming metal occurred. However, this response seemed more subtle and dose dependent for the essential metals while it was more abrupt and long lasting for the non-essential Cd, possibly reflecting its higher potential toxicity. In contrast, actual measured disturbances were highest for Cu, but as this also resulted in more defence mechanisms being activated (e.g. MT and GR), fish could avoid more deleterious effects such as mortality. The results obtained on ion homeostasis for the Cu exposure are in agreement with previous studies showing a disturbance in Na content. However common carp seem to try to cope with this situation through an increased gene expression of H^+-ATPase and Na^+/K^+ ATPase. This is in contrast with Cd, where except for MT induction, we observed little response despite the clear Cd accumulation. This is in line with the results from the 96h-LC_{50} trials which also showed either 0 or 100% mortality without many signs of distress before mortality occurred (Delahaut et al. 2020). It seems that Cd is tolerated up to a certain threshold and then fish die quickly. As these experiments are done at comparable toxicity levels, both Zn and Cd must have affected other physiological processes that were not picked up by our measurements. Therefore, future studies should look at genome wide expression profiling.

Acknowledgments

We are grateful to Steven Joosen for his help with metal and electrolyte analyses, 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 and BS.

Declarations of interest: none

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