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Castaldo, G. *; Pillet, M.; Slootmaekers, B.; Bervoets, L.; Town, R. M.; Blust, R.; and De Boeck, G.

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

*Corresponding author at: Systemic Physiological and Ecotoxicological Research, Department of Biology, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerp, Belgium.

E-mail address: Giovanni.Castaldo@uantwerpen.be

Abstract:

The aquatic environment is continuously under threat because it is the final receptor and sink of waste streams. The development of industry, mining activities and agriculture gave rise to an increase in metal pollution in the aquatic system. Thus a wide occurrence of metal mixtures exists in the aquatic environment. The assessment of mixture stress remains a challenge considering that we can not predict the toxicity of a mixture on the basis of single compounds. Therefore the analysis of the effects of environmentally relevant waterborne mixtures is needed to improve our understanding of the impact of metal pollution in aquatic ecosystems. Our aim was to assess whether 10% of the concentration of the 96h LC_{50} (the concentration that is lethal to 50% of the population in 96h) of individual metal exposures can be considered as a “safe” concentration when applied in a trinomial mixture. Therefore, common carp were exposed to a sublethal mixture of Cu 0.07 ± 0.001 µM (4.3 ± 0.6 µg/L), Zn 2.71 ± 0.81 µM (176.9 ± 52.8 µg/L) and Cd 0.03 ± 0.0004 µM (3.0 ± 0.4 µg/L) at 20°C for a period of one week. Parameters assessed included survival rate, bioaccumulation and physiological biomarkers related to ionoregulation and defensive mechanisms such as MT induction. Our results showed a sharp increase in Cu and Cd concentration in gills within the first day of exposure while Zn levels remained stable. The accumulation of these metals led to a Na drop in gills, liver and muscle as well as a decreased K content in the liver. Biomarkers related to Na uptake were also affected: on the first day gene expression for H^+-ATPase was transiently increased while a concomitant decreased gene expression of the Na^+/H^+ exchanger occurred. A fivefold induction of metallothionein gene expression was reported during the entire duration of the experiment. Despite the adverse effects on ionoregulation all fish survived, indicating that common carp are able to cope with these low metal concentrations, at least during a one week exposure.

Keywords: *Cyprinus carpio*; metal pollution; mixture stress; ionoregulation; ion-homeostasis.

1. Introduction:

The main receptor of anthropogenic discharges is the aquatic ecosystem. Activities such as mining and application of pesticides lead to an increase of metals in the water. Some metals such as copper (Cu) and zinc (Zn) are considered essential because they are important components of enzymes or metalloproteins. Other metals, such as cadmium (Cd), are considered as non-essential metals because they have no role in biological systems (Kalay and Canli 2000).

Fish living in a polluted environment can accumulate these metals via food, or via direct uptake from water through the gills (Perera et al. 2015). Generally, as the concentration of metals increases in the environment, fish accumulate higher levels in their tissues (Al-Attar 2005). When the intake is not balanced with excretion processes and detoxification mechanisms, metals can show their toxic effects (Handy 2003). Gills, considering that they are in direct contact with the aquatic environment, are the main entrance for dissolved substances (Heath 1995). These substances can subsequently reach different organs such as the liver, which is the main organ for metal detoxification, through the circulatory system. When the carrying capacity of the liver is exceeded, they can be stored in other tissues such as muscle. Metal accumulation in the muscle is generally low because it is not a metabolically active tissue, but it is important in transferring the metals through the food chain (Tunçsoy and Erdem 2014).

Metals present in the aquatic environment can be taken up via common uptake routes, interact with each other, and this interaction can affect bioaccumulation and toxicity (Komjarova and Blust 2009). For example Cd and Zn have a comparable electron configuration and a high affinity for molecules containing -SH groups, therefore a competition between $Zn^{2+}$and Cd^{2+} ions is expected for the uptake (Brzóska and Moniuszko-Jakoniuk 2001). Moreover Cd uptake can also be reduced by the presence of Cu (Komjarova and Blust 2009). This reduction in Cd uptake in presence of other metals has been reported by several authors in several species. For example Stewart (1999) reported a reduction of Cd accumulation in the presence of Cu, Zn, Pb and Ni in by a freshwater mussel (*Pyganodon grandis*). Moreover a reduced Cd internal concentration has been found in a freshwater green alga *Chlorella* sp when exposed to a mixture of Cd and Cu (Franklin et al. 2002). A cadmium decrease in presence of copper has also been observed in *Daphnia magna*, in zebrafish and in rainbow trout (Komjarova and Blust 2009, Kamunde and MacPhail 2011, Komjarova and Bury 2014).

Copper is an essential element which is required for several metabolic functions. This metal is involved in bone and tissue formation and considering that it is an enzyme cofactor, it has a role in cellular respiration as well (Pena et al. 1999, Tunçsoy and Erdem 2014). However, when present at high concentrations, Cu can interfere with ionoregulation and increase plasma ammonia, disturbing the acid-base balance as was seen in gibel carp (*Carasius auratus gibelio*) and common carp *(Cyprinus carpio)* (De Boeck et al. 2007). Copper disturbance on ionoregulation is due to its ability to decrease the Na^+/K^+-adenosine triphosphate (Na^+/K^+-ATPase) activity (De Boeck et al. 2001, Wilson and Taylor 1993). The uptake of Cu can be facilitated by a putative Na^+-channels located on the branchial epithelial cells. Moreover an additional sodium (Na^+) uptake pathways present in the gills could be a possible target for Cu-induced inhibition of Na^+ uptake. These uptake pathways are the apical Na^+/H^+ exchanger isoforms (mainly NHE-2 and NHE-3) (Grosell 2011). Thus, the presence of Cu can lead to a competition for the Na^+ uptake sites which results in a decreased Na^+ level (Grosell and Wood 2002, Mackenzie et al. 2004, Niyogi et al. 2015).

Zinc, just as Cu, is an essential element which plays a crucial role in cellular homeostasis, immune responses and oxidative stress (Zhao et al. 2014). The ability of Zn to alter ion-homeostasis has already been demonstrated on different fish such as Nile tilapia (Atli and

Canli 2011) and galaxiid fish (McRae et al. 2016). It has been demonstrated that Zn exposure can alter ion homeostasis as a result of changes in calcium (Ca^{2+}) influx kinetics, inhibition of Ca^{2+}-ATPase and competition with Ca^{2+} for the uptake channel (Hogstrand et al. 1995, McGeer et al. 2000b).

Also Cd constitutes a threat to fish because of its presence worldwide in the aquatic environment and unlike Cu and Zn it is a non-essential metal. Gills are the uptake site for waterborne Cd and it has been demonstrated that it can alter ion-homeostasis in common carp and trout through direct competition with Ca^{2+} at the uptake site (Verbost et al. 1989, Reynders et al. 2006a). In addition Suresh et al. (1995) demonstrated the ability of Cd, in common carp, to reduce Na^{+}, Ca^{2+} and K^{+} levels.

Therefore, all three metals (Cu, Cd and Zn) have in common that they are present worldwide and can interfere with ion-homeostasis in different ways.

Although there are several studies describing the adverse effects of waterborne metals, it is difficult to find information on the effects of metal mixtures. We chose a sublethal mixture of Cu, Zn and Cd at a low concentrations (Cu: 0.08 µM; Cd: 0.03 µM and Zn: 3.16 µM) which represent approximately 10% of the 96h LC_{50} (the concentration that is lethal to 50% of the population in 96h) earlier determined in our lab under the same exposure conditions (96h LC_{50} Cu: 0.77 µM; Cd: 0.20 µM and Zn: 30 µM) (Delahaut et al, 2019a). An initial search of the 96h LC_{50} values found in the EPA ecotox database (EPA 2019) for these metals showed a high degree of variation according to the size, age and water chemistry. The 96h LC_{50} values for Cu ranged from 0.6 to 542 µM (Deshmukh and Marathe 1980, Ganesh et al. 2000), for Zn from 6.9 to 461 µM (Alam and Maughan 1992, Radhakrishnaiah et al. 1993) and for Cd from 0.04 to 862 µM (Kaur and Bajwa 1987, Witeska et al. 1995).

The 10% of the LC_{50} value sometimes is considered as a relatively safe concentration for the organism, at least in a single exposure scenario. According to the 'classic' review by Sprague (1971) a pollutant safe concentration can be estimated by multiplying the LC_{50} values with an application factor of 0.1 (10% LC_{50}) to obtain a concentration which presumably has no sublethal or chronic effects, and these levels have been shown to allow the occurrence of fish populations in the field. Nevertheless, application factors of 0.01 (1% LC_{50}) have also been suggested when looking at reproduction, including for Cu and Zn. Overall, values vary between 0.1-0.4 and 0.01-0.05. According to the US-EPA (US-Environmental Protection Agency), the national recommended acute maximal metal concentrations for the protection of all freshwater aquatic life are 1.84 µM for Zn and 0.02 µM for Cd respectively (EPA 2018) and for Cu the reported value for freshwater corresponds to 0.20 µM (EPA,2004). The guidelines for freshwater surface waters in Flanders, the Belgian region where this study was conducted, impose maximum values of 0.004 to 0.013 µM dissolved Cd depending on water hardness (<40 to >200 mg $CaCO_3$/L) and average dissolved values of 0.31 µM for Zn and 0.11 µM for Cu (Belgian Official Journal, 2015). According to the Flemish Environmental Agency (VMM) the highest measured concentration in 2016 were 88.69 µM for Zn, 2.05 µM for Cu and 1.06 µM for Cd (VMM 2016), clearly exceeding the recommended maximum levels and making our exposure levels highly environmentally relevant.

In the present study common carp, *Cyprinus carpio*, has been chosen as model species for its economic importance worldwide and its use as bioindicator species in environmental pollution studies due its resistance to heavily polluted habitats (Altun et al. 2017, Rajeshkumar et al. 2017). Its availability and ease to handle makes it also a suitable species for transplantation studies for micropollutant bioaccumulation (Bervoets et al. 2009, Schoenaers et al. 2016, Delahaut et al. 2019b). The main question of the present study was: 'Can the 10% of the 96h LC_{50} for Cu, Zn and Cd be considered as a safe concentration when applied in a

mixture?' As mentioned above, an application factor of 0.1 would result in a safe concentration for single metal exposures, however in mixed stress scenario's the different metals could interfere and result in additive or synergistic effects resulting in detrimental effects for the fish. We will answer this question looking at fish survival and metal bioaccumulation, in combination with the assessment of additional physiological parameters determining whether there is an effect on ion-homeostasis, such as on electrolyte loss, induction of Na^+/K^+-ATPase, H^+-ATPase and NHE gene expression, and on defensive mechanisms such as metallothionein (MT) induction, measured as gene expression responses. We hypothesize that the metal mixture remains sub-lethal, but that bioaccumulation will occur and defensive mechanisms will have to be initiated to avoid toxic effects. Even so, we expect that ion-homeostasis will be disturbed, especially for Na and Ca, as the metals use some of the same uptake routes as these ions at the gills.

2. Material and Methods

2.1. Experimental model

Experimental animals, were obtained from the Agricultural University of Wageningen and kept in 1000 L aquaria at 20°C with a photoperiod of 12h light and 12h dark for several months. Three weeks prior starting the experiment, 200 fish were divided in four 200L polyethylene tanks (50 fish per tank) filled with EPA medium-hard water. EPA water 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) (water hardness 0.84 mM or 84.6 ppm $CaCO_3$). Aeration and a biofilter were provided to maintain water quality and the water temperature was maintained at 20°C. Fish were fed with a commercial food (Hikari® Staple™, Klundert, Netherlands) *at libitum* once a day for the whole acclimation period and fasted 2 days prior the start of the experiment. Experimental methods complied with regulation of the Federation of European Laboratory Animal Science Associations and were approved by the local ethics committee, University of Antwerp (Permit Number: 2015-94 Project 32252).

2.2. Experimental set up

Exposures were performed in duplicate and each consisted out of sixty eight months old fish (length = 61.79 ± 9.52 mm; weight = 3.12 ± 0.89 g). Fish were divided between control (EPA medium-hard water) and treatment (EPA medium-hard water containing Cu: 0.08 µM ; Cd: 0.03 µM and Zn: 3.16 µM). Exposure tanks consisted of 5 double-walled polypropylene (PP) buckets for control and 5 double-walled buckets for the treatment, each filled with 9 L of EPA medium-hard water (conductivity 308 ± 2.5 µS/cm) and containing 6 fish. Fish from the first series were used to assess bioaccumulation and electrolytes level, while fish from the second series were used for gene expression analysis and to assess oxidative stress (Pillet et al, 2019). In each bucket, oxygen was provided with an air stone. In order to avoid the accumulation of ammonia and other waste products, 90% of the water was changed daily. Aerated EPA-medium hard water used during the water changes was prepared 24h in advance and kept in the climate chamber at 20 ºC. Water samples were collected before and after water changes to check metal concentration stability. To minimize disturbance to the fish, the perforated inner bucket was lifted from the outer bucket. The fish and 1 L of water stayed behind in the inner bucket and the remaining 8 L of water in the outer bucket could easily be replaced after which the inner bucket was reinserted. The measured metal concentrations during the experiment were Cu 0.003 ± 0.002 µM (0.2 ± 0.1 µg/L), Zn 0.10 ± 0 µM (6.3 ± 0 µg/L), Cd 0.003 ± 0.001 µM (0.3 ± 0.1 µg/L) for the control (N= 140) and Cu 0.07 ± 0.001 µM (4.3 ± 0.6 µg/L), Zn 2.71 ± 0.81 µM (176.9 ± 52.8 µg/L) and Cd 0.03 ± 0.0004 µM (3.0 ± 0.4 µg/L) for the treatment (N= 140). These concentrations correspond to 10% of the 96h LC_{50} previously calculated in our lab from a similar set-up (Delahaut et al., 2019a). Metal speciation was calculated using Visual Minteq.

2.3. Metal accumulation and electrolyte levels in the tissues

On day 1, 3 and 7, ten fish from each treatment (two from each bucket) were euthanized with an overdose of MS222 (pH 7.0, ethyl 3-aminobenzoate methane-sulfonic acid, 300 mg/L, Acros Organics, Geel, Belgium). A muscle sample was cut near the caudal fin, the 1st and 4th gill arch of both left and right side were dissected and pooled per 2 fish to obtain sufficient tissue, as well as the liver and the brain which were collected and pooled per 2 fish. In addition 5 carcasses per treatment and per sampling day were collected to have an overview of the whole body accumulation. The sampled tissues were immediately frozen in liquid nitrogen and stored at -80°C. Metal and electrolyte content in gills, liver, muscle and brain were determined in 5 samples from each tissue (according to the pooled number of samples) at each sampling

time. Samples and reference material (SRM-2976, Mussel tissue, National Institute of Standards and Technology, Gaithersburg, MD, USA) were collected in pre-weighted Eppendorf bullet tubes, dried for 48 hours at 60°C and cooled down in a desiccator for two hours. Subsequently the dry weight (dw) of the samples was recorded with a precision scale (Sartorius SE2, ultra microbalance). Subsequently, the samples were digested by addition of trace metal grade HNO_3 (69%) (Seastar Chemicals) and H_2O_2 (29%) (Seastar Chemicals). The digestion process consisted of 12h digestion at room temperature with HNO_3, followed by a microwave digestion (Blust et al. 1988, Reynders et al. 2006a) of three steps at 100W for three minutes and three steps at 180W for three minutes. At the end of this step H_2O_2 was added followed by a fourth extra microwave digestion step at 300W for two minutes. Metal content and electrolyte levels in the remaining fish carcasses were determined in a similar way. Samples were collected in pre-weighted 50 mL Falcon tubes. Trace metal grade HNO_3 (69%) (Seastar Chemicals) and H_2O_2 (29%) (Seastar Chemicals) was added to the samples. Digestion process started at room temperature for 12 hours, followed by a 30 minute in a hot block (Environmental Express, Charleston, SC, USA) at 100°C. At the end of the process, digested samples were diluted to a final acid concentration of 2% with ultrapure Milli-Q (MQ) water. 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

Ten fish for each treatment from the second series were sacrificed as described above. All the gill arches from each fish were collected, pooled and total RNA was extracted from~20 mg of tissues (gills) using Trizol (Invitrogen, Merelbeke, Belgium) following the manufacturer's instructions. 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 RNA was transcribed to cDNA using a Reverse Transcriptase Core kit (Eurogentec, Seraing, Belgium). cDNA quantity and purity was checked using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the samples were separated into aliquots and frozen at -80°C before being processed. Real-time PCR was performed in duplicate in a final volume of 20 µl using a Mx3000P QPCR System (Agilent Technologies, Belgium). Real-time PCR mastermix contained 10 µL of Brilliant III Ultra-Fast QPCR Master Mix (Agilent), 500 nM of each primer (reverse and forward), 5.7 µL of sterile water, 0.3 µl of reference dye and 5 ng of cDNA. PCR amplification was carried out following the Brilliant III Ultra-Fast QPCR Master Mix (Agilent) protocol for Agilent Mx3000P QPCR system. The relative gene expression of H^+-ATPase, NHE-2 like, Na^+/K^+-ATPase and metallothionein was measured. Six samples were selected according to the OD260/OD280 nm absorption ratio (higher than 1.8) and used for qPCR. As reference gene β-actin and EF1α were used. Oligonucleotides primers for Na^+/K^+-ATPase and for NHE-2 likewere designed using NCBI resources Primer blast and synthesized as highly purified salt-free "OliGold" primers by Eurogentec (Eurogentec, Seraing, Belgium). Primer sequences and accession numbers can be found in table 1.

Gene	accession number	primer 5'--> 3'	Tm °C	Efficiency %
EF1α	Sinha et al. (2012)	F - TGGAGATGCTGCCATTGT	60	92
		R - TGCAGACTTCGTGACCTT	60	
β-actin	Wu et al. (2014)	F - CGTGATGGACTCTGGTGATG	58	96

		R - TCGGCTGTGGTGGTGAAG	62	
Na^+/K^+-ATPase	JX570881.1	F - ATGGGTCGTATCGCCACTCT	62	104
		R - CCAGGAAGACAGCAACACCA	62	
NHE-2	XM_019098528	F – CACACAAGCTTACGACGCAG	59.8	107
		R - TCCAGTGTGAACGAGTCTCC	59	
H^+-ATP	Sinha et al. (2016)	F - CTATGGGGGTCAACATGGAG	62	103
		R - CCAACACGTGCTTCTCACAC	62	
Metallothionein	Reynders et al. (2006b)	F - CCAAGACTGGAACTTGC	52	93
		R - ACGTTGACCTCCTCAC	50	

Table 1: Primer sequences (F= forward; R= reverse) and TM°C of target and housekeeping genes.

2.5. Statistical analysis

All data have been presented as mean values ± standard deviation (S.D.). Normality was verified by the Shapiro-Wilk test. For comparisons between different experimental groups a two-way analysis of variance (ANOVA) was performed followed by Tukey test using GraphPad Prism version 7.04 for Windows (GraphPad Software, La Jolla California USA).

3. Results

No mortality and no adverse behaviour were observed during the experiment. The speciation of metal ions calculated in Visual Minteq resulted in free metal ion concentration, expressed in µM, of Cu^{2+} 0.004, Cd^{2+} 0.02 and Zn^{2+} 2.07. The species distribution in our media showed approximately 7% of Cu^{2+}, 76% Zn^{2+} and 85% Cd^{2+}. More information can be found in supplementary information table (SI) 1 and 2. Non-significant results of metal accumulation and electrolyte levels not shown in the graphs below can be found in the SI-table 5.

3.1. Metal accumulation

3.1.1. Cu accumulation

Copper concentrations in gills, liver, muscle, brain and carcass are shown in fig. 1. A sharp increase of Cu occurred in the gills (Fig. 1A and SI-table 3). A significant increase in treated fish compared to the control fish was already evident after one day (≃ 83%) and a further increase was observed at the end of the experiment (≃ 352%). In addition, at the end of the experiment the concentration in the treatment almost doubled (91%) compared to the treatment at day 3. Copper content in the liver (Fig. 1B) was stable for the first three days and a significant increase (≃ 39%) in the liver compared with the control only took place after one week of exposure. Concentration in muscle and in the brain (respectively Fig. 1C and D) did not show any significant elevation when comparing the control and the exposed group. Differences in Cu content in the remaining carcass (Fig. 1E) only increased significantly at day 7 (≃ 38%). By the end of the experiment, Cu concentration in the treatment compared to the control increased about 90% in the gills, while in the liver and in the carcass the increase was close to 40% (39% and 38% respectively). Thus the accumulation pattern is gills > liver > carcass.

3.1.2. Cd accumulation

Similar to Cu, Cd accumulation in the gills (Fig. 2A and SI-table 3) showed a pronounced increase in the treatment compared with the control at day 1 (≃ 202%), day 3 (≃ 563%) and day 7 (≃ 731). At day 3 the amount of Cd in the treatment was almost three times higher compared with day 1 and a further increase can be observed at day 7 compared to day 3. In the liver (Fig. 2B) no significant differences between control and treatment were observed. Cadmium concentration in the carcass (Fig. 2C) showed a significant increase in the exposed group compared with the control group at day 3 (≃ 79%) and 7 (≃ 105%). In muscle and brain Cd levels remained below the detection limit (see SI-table 5).

3.1.3. Zn accumulation

Concerning Zn accumulation, no statistical differences have been found between control and treatment in any of the analysed tissues. The average values for Zn (nmol/g dw) in the different tissues are: gills control (13336 ± 2438), gills treatment (12192 ± 2202), liver control (4201 ± 794), liver treatment (3724 ± 1205), muscle control (462 ± 107), muscle treatment (509 ± 142), brain control (561 ± 165), brain treatment (655 ± 277), carcass control (5379 ± 1108) and carcass treatment (6502 ± 1720). More details about Zn levels in all the different tissues can be found in the SI-table 3 and 5.

3.2. Electrolyte content

3.2.1. Sodium

Changes in Na homeostasis are shown in fig. 3. In the gills (Fig. 3A and SI-table 4), we observed a significant decrease in Na content (≃ 20%) in the treatment compared to the control from day one until day 7. In the liver (Fig. 3B) as well as in the carcass (Fig. 3D) a difference between control and treatment was observed only after 3 days of exposure (respectively ≃ 35% and 23%). In muscle tissue (Fig. 3C) a Na drop in the treated group occurred at day 3 and at day 7 (≃ 28%). No changes were observed in the brain (see SI-table 5).

3.2.2. Potassium and Magnesium

Potassium (K) content in the liver (Fig. 4A) shows a decrease after 3 and 7 days in the treatment compared to the control (≃ 27%) and compared to the same group at day 1 (≃ 23%). A decrease in magnesium (Mg) between treatment and control in the liver (Fig. 4B) was noticed after three days of exposure (≃ 20%). In the remaining carcass (see SI-table 5) a decrease in K in the treatment compared to the control occurred at day 3 (≃ 13%). Magnesium content in the brain (see SI-table 5) increased significantly in the treatment after seven days, but this was mainly caused by slightly lower, but highly variable level in the control group. No differences were observed both for K and Mg in all the remaining tissues (see SI-table 5).

3.3. Gene expression

3.3.1. Metallothionein

Metallothionein gene expression is shown in fig. 5. It is clear that the gene coding for metallothionein is strongly induced in the exposed group compared to the control from the first day onwards.

3.3.2. Ionoregulation

Relative gene expression of genes coding for Na^+/K^+-ATPase, H^+-ATPase and the NHE-2 in the gills are shown in fig 6. Na^+/K^+-ATPase mRNA expression only showed a weakly decreasing trend over time with no significant differences between control and treatment. However, the expression of the gene coding for the NHE-2 showed a significant decrease due to metal exposure at day 1 and 3 (≃ 30%). In contrast, H^+-ATPase gene expression showed a significant increase after one day of exposure in the treatment compared to the control (≃ 53%) with a recovery to control levels thereafter.

4. Discussion:

As previously mentioned we hypothesized that we expected bioaccumulation and induction of protective mechanisms such as MT. A parallel study showed that antioxidant mechanisms were activated under these exposure conditions in common carp, thus avoiding oxidative stress (Pillet et al. 2019). Therefore, it seems that defensive mechanisms in common carp were able to respond adequately to protect the fish from damage. Nevertheless, even with these protective mechanisms we were expecting negative effects on ion-homeostasis and ionoregulation. Therefore we will first discuss the bioaccumulation in a trinomial mixture including assessing changes in MT gene expression, and secondly the effects of the accumulated metals on disturbances in ionoregulation.

4.1. Dynamics of Cu, Cd and Zn accumulation and MT gene expression.

Our results confirmed that gills and liver accumulated metals earlier and in larger amounts than brain and muscle tissues. Gills accumulated 192 nmol/g dw of Cu, 36 nmol/g dw for Cd and 949 nmol/g dw for Zn. In a study done by Delahaut et al. (2019a) in slightly larger carp exposed 10 % LC_{50} of Cu, Cd and Zn for one week in single exposures, accumulated concentrations were 130 nmol/g dw for Cu, 90 nmol/g dw for Cd and 4610 µmol/g dw for Zn. By extrapolating the results obtained from our own previous work (Castaldo et al; unpublished data) of common carp exposed to 25, 50 and 100% of the LC_{50} value of Cu, Cd and Zn as single exposures, we predicted the net accumulated for fish exposed to 10 % LC_{50} of the above metals. For Cu exposure, the predicted net accumulated metal at day 7 corresponds to 112 nmol/g dw; for Cd to 81 nmol/g dw and for Zn, the predicted level is 4450 nmol/g dw. Comparing results obtained from a single exposure scenario with results obtained in a ternary mixture we can observe that the net accumulation of Cu is slightly higher in the mixture. However, for Cd and Zn the situation is different. In the single exposure scenarios, the net accumulated metal is always significantly higher compared to the mixture scenario. For Cd, the accumulation halved in the mixture, and Zn accumulation was only one fourth. This is also reflected by the accumulation rate for the extrapolated data for Cd (0.5 nmol/g dw/h at day 7), which was always the double of the mixture scenario. We can assume that this difference in the accumulation between the two experimental scenarios is due to the competition between Cu and Cd as already demonstrated in several species, such as the freshwater mussel (*Pyganodon grandis*), *Daphnia magna* and zebrafish (Stewart 1999, Komjarova and Blust 2009, Komjarova and Bury 2014). Similarly, a competition between Cd and Zn which led to a reduced Cd accumulation has been observed in different species, such as the euryhaline black sea bream (*Acanthopagrus schlegeli*) and in zebrafish (Komjarova and Blust 2009). Furthermore in agreement with Brix et al. (2017) who found a reduced Zn uptake in rainbow trout gills exposed to a binary mixture of Zn and Cd/Cu, we found that Zn accumulation in the mixture scenario was 4 times lower in the present study compared to the predicted single exposure scenario. Therefore, we can assume that in common carp, similar to rainbow trout (Brix et al. 2017), a reciprocal inhibition of metal uptake can occur.

Besides exposure concentration, water parameters such as temperature, water hardness and pH are important for metal uptake and metal toxicity (Witeska and Jezierska 2003) as they determine metal speciation. In our study, we generally found a clear and sharp increase in Cu and Cd bioaccumulation especially in gills, while no significant accumulation was found for Zn in any of the tissues. We have to take into account that Zn is one of the most abundant ions in the body and it has a key role for the activity of hundreds enzymes (Zhao et al. 2014). Therefore fish have several transporters to control the uptake, excretion and internal Zn homeostasis (Bury et al. 2003). It has previously been demonstrated that rainbow trout and other teleost fish can excrete the exceedance of Zn through bile, intestinal sloughing (Handy

1996) and gills (Hardy et al. 1987). Moreover fish are able to regulate Zn uptake; for instance rainbow trout show an alteration in Zn uptake mechanism in order to reduce the amount of accumulating Zn trough the gills when exposed to high waterborne Zn levels (Bury et al. 2003). This is in accordance with our accumulation rates which show values close to zero.

Cu accumulation in the gills showed a quick and time dependent accumulation. The highest accumulation was observed in the gills, followed by the liver and carcass (gills > liver > carcass). No differences were observed in the remaining tissues. Cu accumulation has been studied by several authors and in some cases, metal accumulation is assessed only after several days of exposure (Dixon and Sprague 1981, McGeer et al. 2000a, De Boeck et al. 2004). Such a fast Cu increase after one day of exposure in the gills was unexpected in the present study considering that our fish were exposed to a very low concentration. However, gills are in direct contact with the external media and are the primary uptake site of the organism under waterborne exposures. Interestingly gill Cu concentration almost doubled at the end of the experiment, even though the accumulation rate decreased compared to the previous days, while the rest of the studied tissues did not show such a substantial difference. This suggests that internal homeostatic mechanisms were acting to maintain Cu homeostasis in equilibrium in the body (Kamunde et al. 2002). However we have to take into account that we did a short term exposure using relatively low exposure concentrations and tissues have high Cu background levels, thus time can play an important role in metal accumulation in these tissues. Considering that liver is the main accumulating organ, we expected a greater accumulation in the liver rather than in the gills by the end of the experiment (De Boeck et al. 2003). A significant difference between control and treatment in the liver was only observed at day 7 in concomitance with the drastic increase of Cu in the gills (see Fig. 1A). This delay in the accumulation can be explained by the important role that the liver plays in Cu homeostasis. In mammals, as well as in freshwater fish such as rainbow trout, Cu excess is excreted by the hepatobiliary system (Grosell 2011). Therefore, this Cu increase in the analysed tissues at the end of the exposure period lets us assume that renal and hepatobiliary excretion were no longer capable to counteract the metal uptake.

Cadmium, in contrast with Zn and Cu, does not have any known biological role and therefore it is considered as a non-essential metal and extremely toxic for fish (Matsuo et al. 2005). Similar to Cu, Cd accumulation mainly occurred in gills. As expected Cd accumulated fast in the gills (Vinodhini and Narayanan 2008) and especially against the low background levels accumulation was significant from the start. Such a fast Cd accumulation in the gill tissue indicate their vulnerability as the main uptake site of metals during waterborne exposures (Benhamed et al. 2016). In the present experiment no significant Cd accumulation occurred in the liver, possibly due to the short exposure time, excretion of the metal via faeces, mucosal sloughing or hepatobiliary excretion (McGeer et al. 2011).

It is well known that metallothioneins are cysteine rich proteins which can bind metals with high affinity (Nordberg 1998). They have a double task, on the one hand MTs can participate in essential metal homeostasis; while on the other hand MT are involved in detoxification processes, sequestering and binding excess of essential and non-essential metals (De Boeck et al. 2003). Several studies have shown the protective role of MT and the relationship between MT levels and metal accumulation in different tissues in single exposures (De Smet et al. 2001, Lange et al. 2002, De Boeck et al. 2003, De Boeck et al. 2010). However, MT induction is not always sufficient as Zhu et al. (2018) found that Cd caused damage at the gill surface despite a MT gene expression increase in mud crab (*Scylla paramamosain*). In our work MT mRNA expression drastically increased in the treatment during the whole exposure period. Usually MTs are present in the tissues in low amounts and when fish are exposed to metals, thionein synthesis is induced (Hamilton and Mehrle 1986). Therefore the fast and

strong induction can be related to the fast accumulation of Cu and Cd in the gill. One can assume that MT synthesis and protein levels are increasing following the induction of gene expression. As mentioned before, for Cu and Zn MTs have a homeostatic role. Metallothionein can both sequestrate essential metals and donate them for biochemical reactions (Roesijadi 1992). The sudden increase in MT mRNA expression corresponds well with the immediate increase in gill Cd. Similar results have been found in goldfish injected with $CdCl_2$ for 36h and coho salmon where the presence of Cd strongly enhanced the expression of MT in different tissues (Choi et al. 2007, Espinoza et al. 2012). Therefore we hypothesize that first a depletion of available MT and of glutathione (GSH) already present in the tissue, which both acts as first line of defence complexing metals before MTs synthesis is induced, took place (Lange et al. 2002). Secondly, considering that expression of the MT gene remained induced in the treatment compared to the control, we hypothesize that continuous MT production was necessary to cope with waterborne Cu and Cd uptake. Moreover, the resulting MT levels seemed to be able to bind a sufficient amount of metals, at least for one week, to avoid more deleterious effect which can lead to mortality. Metallothionein binding affinity changes according to the metal, for example in presence of Cd the essential metal associated with the MT is displaced (Amiard et al. 2006). In vitro studies demonstrated that the protein binding affinity decreases in the hierarchical sequence $Hg^{2+} > Cu^{+}$, Ag^{+}, $Bi^{3+} \gg Cd^{2+} > Pb^{2+} > Zn^{2+} > Co^{2+}$ (Vašák 1991). Therefore in presence of Cd and Cu, Zn is displaced from the MT. This Zn displacement could have allowed the MT to bind the more toxic compounds such as Cu and Cd in our study, thus protecting the fish.

4.2. Effects of metal exposure on ionoregulation.

Ions such as Na^{+}, K^{+}, Mg^{2+} and electrolytes in general are important for the physiological and metabolic process of the organism. For instance Na^{+} is one of the major cations of the extra-cellular fluid, while K^{+} is the major cation of the intracellular environment (Sathya et al. 2012). Our results showed a decrease in total Na in almost all the analysed tissues. Gills are the most affected tissue as previously reported in several other studies (De Boeck et al. 2001, Grosell and Wood 2002, Mackenzie et al. 2004, Niyogi et al. 2015). In our results the decrease in Na occurs in concomitance with Cd and especially Cu bioaccumulation in the gills. Therefore, competition between Cu and Na at the uptake site could explain this trend (Niyogi et al. 2015). In order to better understand this Na decrease we analysed the expression of the Na^{+}/K^{+}-ATPase, NHE-2 and H^{+}-ATPase genes. 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). Moreover Na can enter in the body through gill epithelia through a H^{+} exchanger or through an ion channel coupled with the apical H^{+}-ATPase (McCormick 2001) which provide the driving force creating an electrochemical gradient for Na uptake in freshwater fish (Lin and Randall 1993). Our results only showed a trend towards reduced Na^{+}/K^{+}-ATPase mRNA suggesting that metals, at such a low concentration, are acting more at protein level rather than at molecular level, inhibiting the Na^{+}/K^{+}-ATPase at the Mg^{2+} binding site (Li et al. 1996). Regarding the NHE there is a drop in mRNA abundance in the treatment after one and three days of exposure, while for the H^{+}-ATPase there is an increase in the gene expression. Several NHEs have been identified and the NHE-2 is the candidate for the Na-sensitive component of Cu uptake in zebrafish gills (Mackenzie et al. 2004, Craig et al. 2010, Komjarova and Bury 2014). Our results showed a reduced NHE gene expression in concomitance with the sodium reduction. This could be explained in two ways, or as an indirect effect of Cu on the carbonic anhydrase activity which lead to a depletion of H^{+} as described by Grosell (2011) or as an attempt of the fish to reduce Cu influx which is affecting Na concentrations as well. However, the organism seems to recognize a disturbance in ion-homeostasis and it might attempt to compensate by increasing the expression of the gene coding for H^{+}-ATPase. This hypothesis is supported by

the evidence that the gills are not showing any further Na decreases. However the general Na loss in the gills led to a Na drop in liver and muscle as well (Fig. 3B and 3C).

Potassium homeostasis has been investigated by several authors in different species and with inconsistent results. For example Cerqueira and Fernandes (2002) investigated the effects of Cu in *Prochilodus scrofa* on blood parameters finding an increase in plasma K after Cu exposure to 0.46 µM, while Atli and Canli (2011), found a Na^+ and K^+ decrease in *Oreochromis niloticus* tissues individually exposed to Cu, Cd and Zn. Our results showed a total K decrease in the liver. Ions are important for maintaining cell volume and the exchange between Na^+ and K^+ powered by Na^+/K^+ pump is necessary to prevent cell swelling (Lodish et al. 2000). This electrolyte loss can probably be related to the ability of metals to inhibit the ion-transporting enzymes (Suresh et al. 1995, McGeer et al. 2000b, Matsuo et al. 2005) and can lead to cell damage. The ability of Zn and Cd to cause hypocalcaemia has been reported by several authors (Verbost et al. 1989, Hogstrand and Wood 1995, McGeer et al. 2000b). However in most of these studies fish are exposed to higher metal concentrations (Atli and Canli 2011). Surprisingly our results show no alteration in Ca level. This could be due to a lack of Zn accumulation, relatively low waterborne Cd concentrations and the short exposure period.

5. Conclusions

The used concentrations represent a sublethal concentration when considered as single compound and as a mixture, but do not qualify as a No Observed Effect Concentration (NOEC) level as a mixture. Our results showed the ability of the metal mixture to interfere with ionoregulation despite a pronounced induction in MT gene expression. Further Na losses appeared to be prevented by the ability of common carp to cope with this situation through an increased expression of the H^+-ATPase gene. In a parallel experiment Pillet et al. (2019) showed the ability of common carp to avoid oxidative stress when exposed at the same mixture. When exposed to a mixture of Cu, Cd and Zn at a concentration of 10% of the LC_{50}, the bioaccumulation pattern is similar to those described in other papers with sharp increases in Cu and Cd (De Smet et al. 2001, Celechovska et al. 2007, Tunçsoy and Erdem 2014) followed by a drop in Na. However most of the studies only describe the accumulation of a single metal, often at a higher concentration. Many of the observed effects in our study can be attributed to Cu rather than Cd or Zn, indicating its toxic capacities, even at low concentrations. Whereas Cu accumulation might have been slightly increased due to the mixture effect, there was a clear antagonistic effect on Cd and Zn accumulation. In conclusion, our results indicate that trace metals mixtures can easily affect the life of fresh water fish. Nevertheless our results and the absence of mortality suggested that the fish are able to cope with this situation at least for one week and that recovery processes are rapidly acting to protect the fish. Further studies using longer exposures will be conducted to test this hypothesis.

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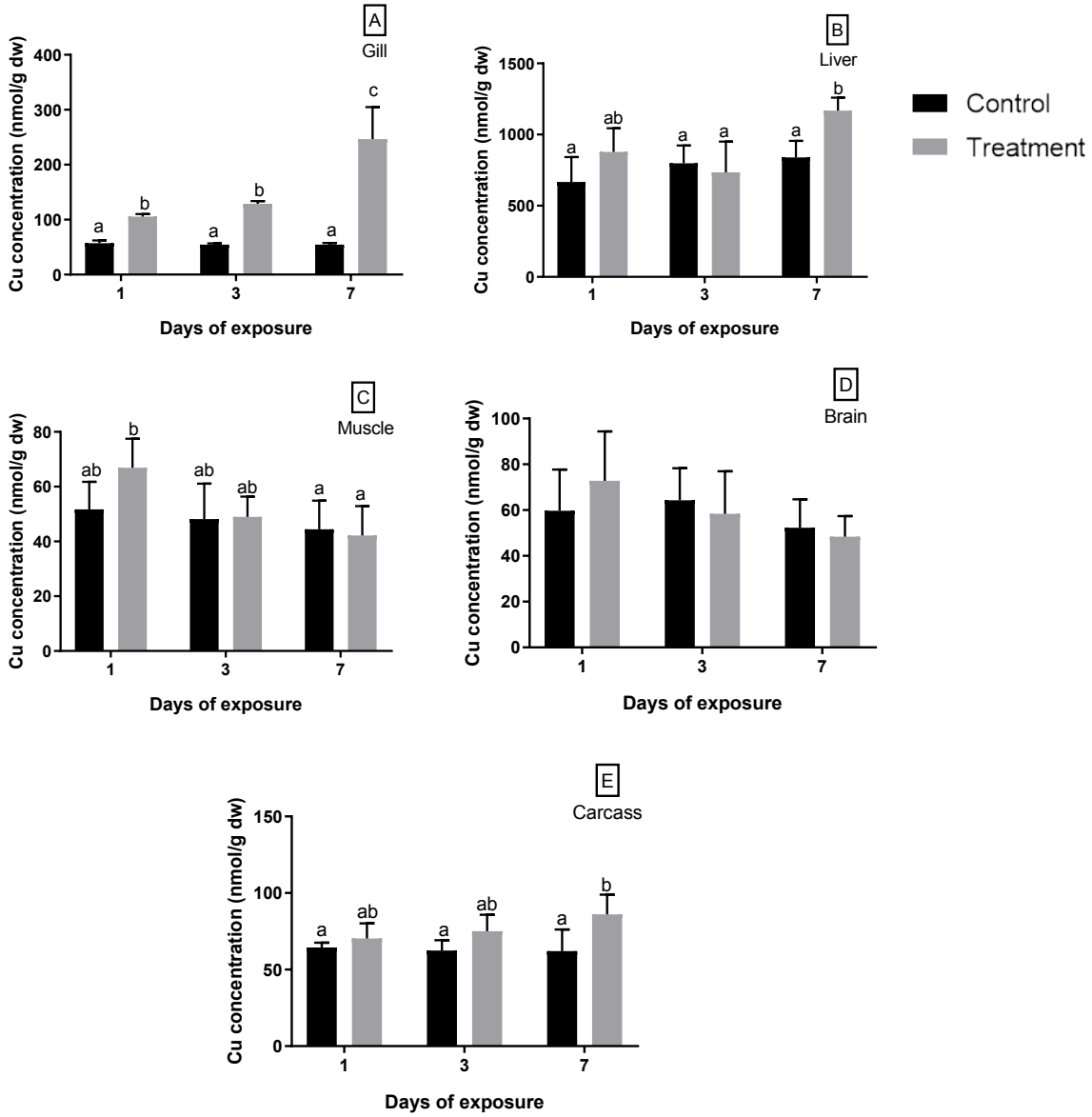

Figure 1: Copper concentration (nmo/g dw) in gills (A), liver (B), muscle (C), brain (D) and carcass (E) of *Cyprinus carpio* exposed to Cu/Cd/Zn mixture for 1, 3 and 7 days (mean ± standard deviation). Two-way analysis of variance (ANOVA) was performed followed by Tukey. Mean ± SD, N=5, letters indicate significant differences ($p < 0.05$).

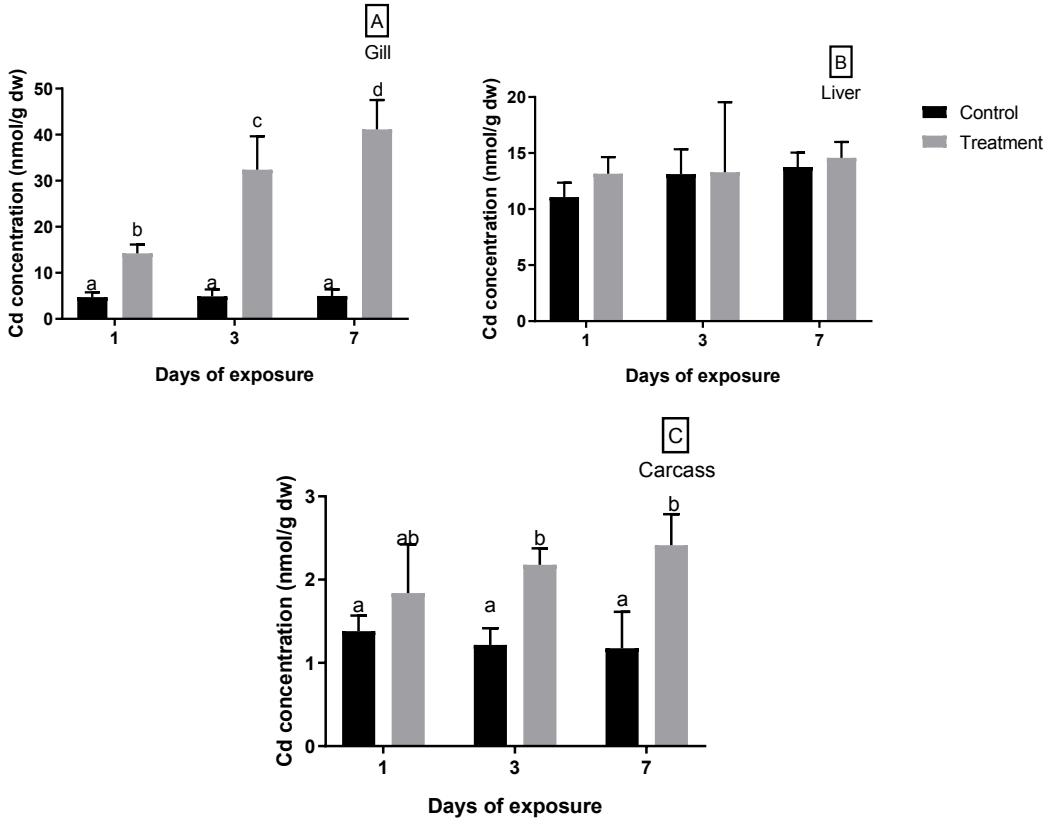

Figure 2: Cadmium concentration (nmol/g dw) in gills (A), liver (B), carcass (C) of *Cyprinus carpio* exposed to Cu/Cd/Zn mixture for 1, 3 and 7 days (mean ± standard deviation), results for muscle and brain were BMQL and are not showed. Two-way analysis of variance (ANOVA) was performed followed by Tukey. Mean ± SD, N=5 , letters indicate significant differences ($p < 0.05$).

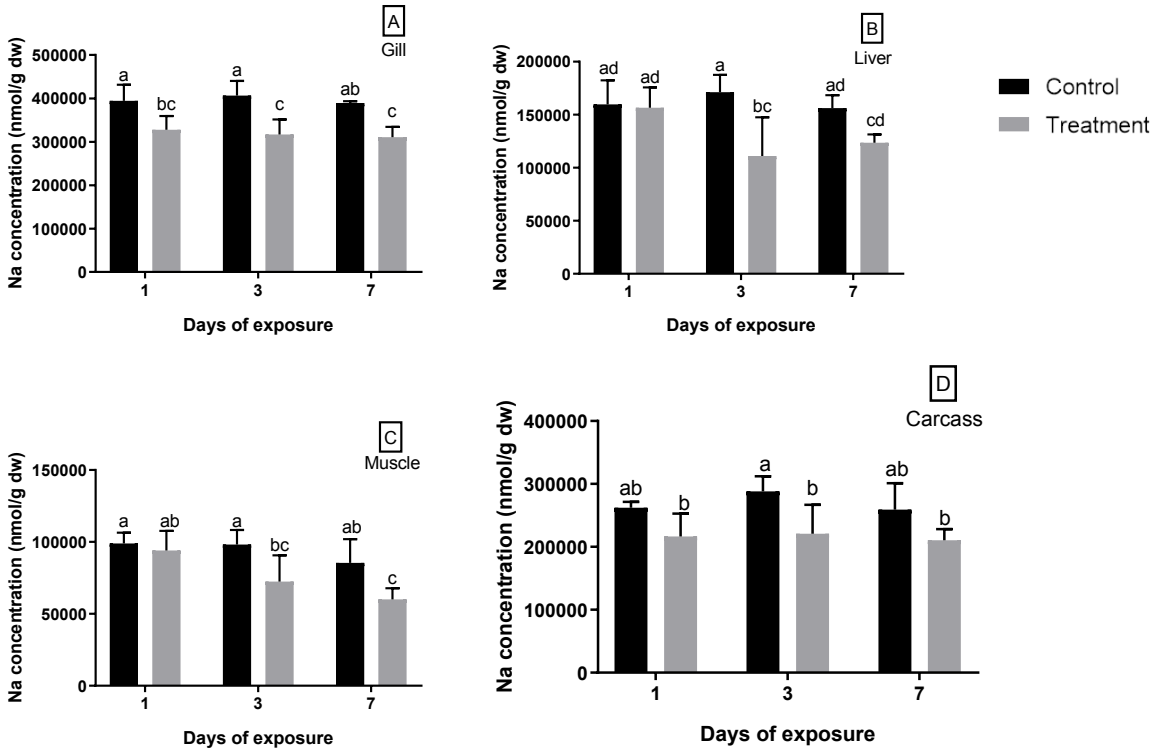

Figure 3: Sodium concentration (nmol/g dw) in gills (A), liver (B),muscle (C), carcass (D) of *Cyprinus carpio* exposed to Cu/Cd/Zn mixture for 1, 3 and 7 days (mean ± standard deviation). Two-way analysis of variance (ANOVA) was performed followed by Tukey. Mean ± SD, N=5 , letters indicate significant differences ($p < 0.05$).

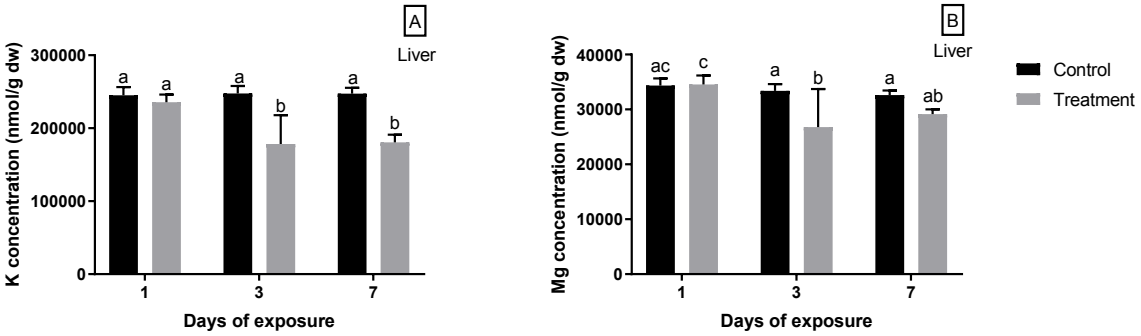

Figure 4: Liver potassium (A) and magnesium (B) concentration (nmol/g dw) of *Cyprinus carpio* exposed to Cu/Cd/Zn mixture for 1, 3 and 7 days (mean ± standard deviation). Two-way analysis of variance (ANOVA) was performed followed by Tukey. Mean ± SD, N=5 , letters indicate significant differences ($p < 0.05$).

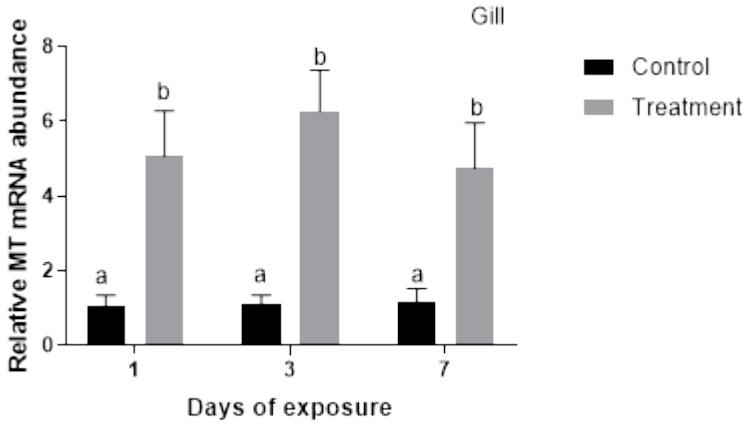

Figure 5: Relative genomic expression of gene coding for metallothionein in gills of *Cyprinus carpio* exposed to Cu/Cd/Zn mixture for 1,3 and 7 days (mean ± standard deviation). Two-way analysis of variance (ANOVA) was performed followed by Tukey. Mean ± SD, N=6 , letters indicate significant differences ($p < 0.05$).

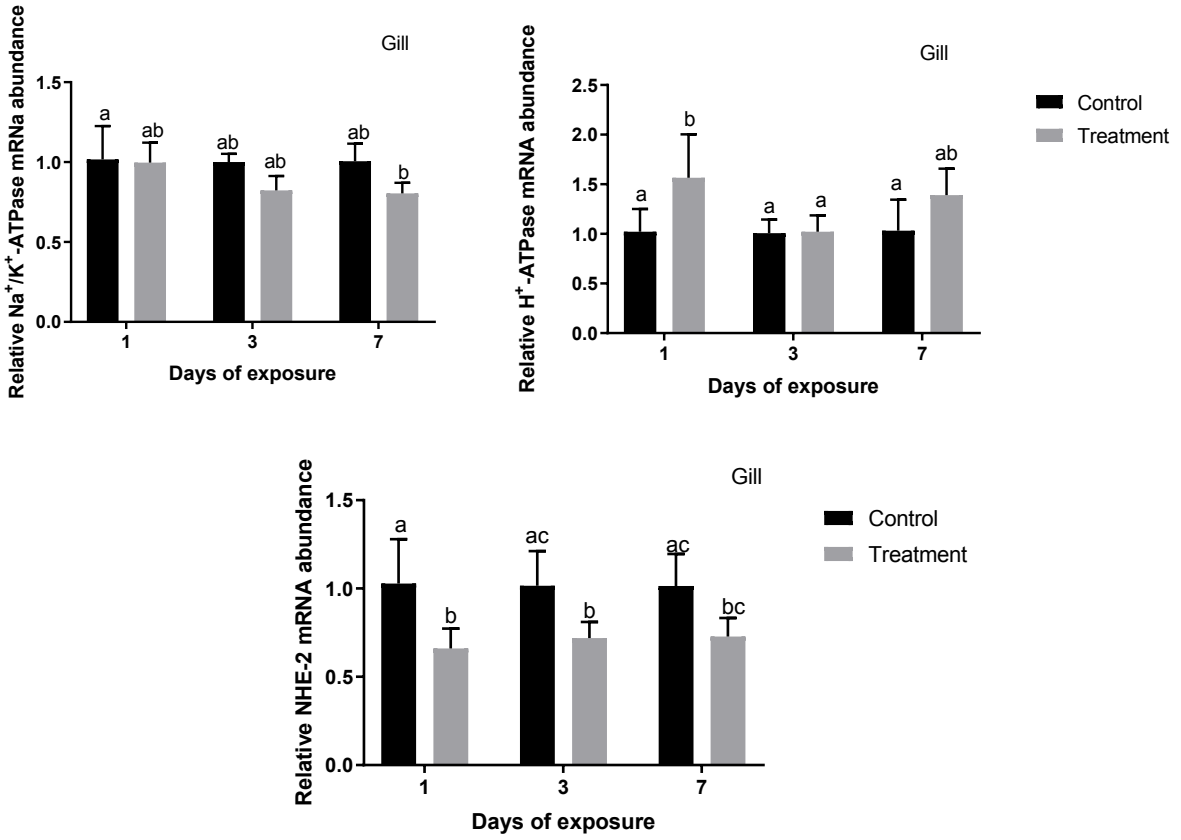

Figure 6: Relative genomic expression of gene coding for Na^+/K^+-ATPase, H^+-ATPase and NHE-2 in gills of *Cyprinus carpio* exposed to Cu/Cd/Zn mixture for 1,3 and 7 days (mean ± standard deviation). Two-way analysis of variance (ANOVA) was performed followed by Tukey. Mean ± SD, N=6 , letters indicate significant differences ($p < 0.05$).

Supplementary tables

SI-table 1: Metal speciation in the media calculated with Visual Minteq taking into account one single metal at time.

Component	Concentration	% of total concentration	Species name
Cu^{+2}	4.7880E-09	6.840	Cu^{+2}
		8.709	$CuOH^{+}$
		0.638	Cu(OH)2 (aq)
		0.649	CuSO4 (aq)
		82.048	CuCO3 (aq)
		0.319	$CuHCO3^{+}$
		0.788	$Cu(CO3)2^{-2}$
Zn^{+2}	2.0733E-06	76.507	Zn^{+2}
		0.011	$Zn(CO3)2^{-2}$
		2.684	$ZnOH^{+}$
		2.936	Zn(OH)2 (aq)
		7.050	ZnSO4 (aq)
		0.049	$Zn(SO4)2^{-2}$
		8.965	ZnCO3 (aq)
		1.788	$ZnHCO3^{+}$
Cd^{+2}	2.5513E-08	85.045	Cd^{+2}
		0.239	$CdOH^{+}$
		0.302	$CdCl^{+}$
		8.257	CdSO4 (aq)
		0.091	$Cd(SO4)2^{-2}$
		1.993	$CdHCO3^{+}$
		4.061	CdCO3 (aq)
		0.011	$Cd(CO3)2^{-2}$

SI-table 2: Metal speciation in the media calculated with Visual Minteq taking into account all the three metals together.

Component	Concentration	% of total concentration	Species name
Cu^{+2}	4.79E-09	6.842	Cu^{+2}
		8.712	$CuOH^{+}$
		0.638	Cu(OH)2 (aq)
		0.649	CuSO4 (aq)
		82.044	CuCO3 (aq)
		0.319	$CuHCO3^{+}$
		0.788	$Cu(CO3)2^{-2}$
Zn^{+2}	2.07E-06	76.508	Zn^{+2}
		0.011	$Zn(CO3)2^{-2}$
		2.684	$ZnOH^{+}$
		2.936	Zn(OH)2 (aq)
		7.05	ZnSO4 (aq)
		0.049	$Zn(SO4)2^{-2}$
		8.965	ZnCO3 (aq)
		1.788	$ZnHCO3^{+}$
Cd^{+2}	2.55E-08	85.05	Cd^{+2}
		0.239	$CdOH^{+}$
		0.302	$CdCl^{+}$
		8.254	CdSO4 (aq)
		0.091	$Cd(SO4)2^{-2}$
		1.992	$CdHCO3^{+}$
		4.06	CdCO3 (aq)
		0.01	$Cd(CO3)2^{-2}$

SI-table 3: Metals net accumulation, *% of increase and accumulation rate in the gills of common carp exposed to a metal mixture for 1, 3 and 7 days.*

Gills									
	Copper			Zinc			Cadmium		
Exposure Day	Day 1	Day 3	Day 7	Day 1	Day 3	Day 7	Day 1	Day 3	Day 7
Net accumulation (nmol/g dw)	48 ± 4	74 ± 5	192 ± 58	-2801 ± 2365	- 1579 ± 3268	949 ± 1350	9 ± 2	27 ± 7	36 ± 6
% of increase	83 ± 8	135 ± 9	352 ± 107	-18 ± 15	-12 ± 24	8 ± 12	202 ± 40	562 ± 147	730 ± 127
accumulation rate (nmol/g dw/h)	2 ± 0.2	1 ± 0.1	1 ± 0.3	-117 ± 98	-22 ± 45	6 ± 8	0.4 ± 0.1	0.4 ± 0.1	0.2 ± 0.04

SI-table 4: Sodium net loss, % of loss and loss rate in the gills of common carp exposed to a metal mixture for 1, 3 and 7 days.

Gills			
	Sodium		
Exposure Day	Day 1	Day 3	Day 7
Net Na loss (nmol/g dw)	66819 ± 31763	89408 ± 34463	78558 ± 23878
% Na loss	17 ± 8	22 ± 8	20 ± 6
Rate of Na loss (nmol/g dw/h)	2784 ± 1323	1241 ± 478	467 ± 142

SI-table 5: Metal content and electrolytes level (nmol/g dw) in gills, liver, muscle, brain and carcass of common carp at day 1, 3 and 7. Mean ± SD, N=5, letters indicate significant differences ($p < 0.05$).

	Day 1		Day 3		Day 7	
Gills	Control	Treatment	Control	Treatment	Control	Treatment
Cu	57.3 ± 4.7 [a]	105.4 ± 4.6 [b]	54.57 ± 2.2 [a]	129 ± 5.2 [b]	54.5 ± 3 [a]	246.4 ± 58.3 [c]
Zn	15212 ± 2336	12410 ± 2364	13637 ± 1511	12057 ± 3268	11160 ± 2032	12109 ± 1351
Cd	4.7 ± 1.1 [a]	14.2 ± 1.9 [b]	4.9 ± 1.5 [a]	32.4 ± 7.2 [c]	4.9 ± 1.4 [a]	41.1 ± 6.3 [d]
Na	394392 ± 36979 [a]	327573 ± 31763 [b]	406298 ± 33897 [a]	316890 ± 34463 [b]	389203 ± 4421 [a]	310644 ± 23878 [b]
K	287778 ± 38763	270985 ± 8234	273472 ± 19587	269887 ± 6423	277264 ± 12174	263396 ± 10052
Mg	64283 ± 6663	60721 ± 3783	67574 ± 5045	60026 ± 4573	63223 ± 3465	64050 ± 6106
Ca	885485 ± 88395	836640 ± 80914	958033 ± 73731	841649 ± 98356	955092 ± 75227	928377 ± 98761
Liver						
Cu	667 ± 175 [a]	878 ± 166 [ab]	799 ± 124 [a]	735 ± 216 [a]	840 ± 116 [a]	1168 ± 91 [b]
Zn	4309 ± 883.8	4433 ± 1207	4257 ± 908	3180 ± 1708	4037 ± 840	3452 ± 649
Cd	11.1 ± 1.3	13.16 ± 1.5	13.1 ± 2.2	13.3 ± 6.2	14 ± 1.3	14.6 ± 1.4
Na	159782 ± 22712 [ad]	156621 ± 19097 [ad]	171188 ± 16426 [a]	111050 ± 36462 [bc]	156250 ± 12247 [ad]	123592 ± 7825 [cd]
K	245121 ± 11203 [a]	235685 ± 10519 [a]	247699 ± 10132 [a]	178419 ± 39540 [b]	247431 ± 7953 [a]	180570 ± 10602 [b]
Mg	34341 ± 1305 [ac]	34557 ± 1613 [c]	33397 ± 1227 [a]	26769 ± 6955 [b]	32617 ± 821 [a]	29147 ± 869 [a]
Ca	8917 ± 5704	6240 ± 112	5916 ± 1521	5248 ± 3846	4822 ± 225	3778 ± 225
Muscles						
Cu	51.7 ± 10.1 [ab]	66.9 ± 10.6 [b]	48.1 ± 13 [ab]	49 ± 7.3 [ab]	44.4 ± 10.5 [ab]	42.1 ± 10.8 [a]
Zn	469 ± 90	647 ± 190	504 ± 145	462 ± 96	414 ± 90	438 ± 66
Cd	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL
Na	98937 ± 7501	94022 ± 13665	98185 ± 10163	72480 ± 18207	85447 ± 16448	60009 ± 7817
K	437932 ± 33580	463034 ± 55073	444584 ± 29027	473078 ± 32422	413152 ± 45318	443479 ± 64765
Mg	64755 ± 4770	72106 ± 8203	68055 ± 6323	70363 ± 1656	61472 ± 6575	68195 ± 8898
Ca	39983 ± 13065 [a]	48458 ± 13077 [ab]	42637 ± 9297 [a]	54271 ± 24748 [bc]	37548 ± 8197 [ab]	58757 ± 16589 [c]
Brain						
Cu	59.7 ± 18	72.9 ± 21.6	64.3 ± 14	58.35 ± 18.6	52.3 ± 12.5	48.4 ± 9
Zn	536.8 ± 173	850.4 ± 436.4	676.5 ± 195.7	605.2 ± 98.55	493.3 ± 128.7	510.8 ± 103.1
Cd	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL
Na	252521 ± 15272	224559 ± 55703	255698 ± 11567	249265 ± 11753	256150 ± 48805	245486 ± 7503
K	380905 ± 14244	398930 ± 10205	394387 ± 15238	382459 ± 9524	389713 ± 8135	385095 ± 8510
Mg	29904 ± 2066 [a]	32411 ± 781 [a]	30413 ± 899 [a]	31390 ± 489 [a]	28675 ± 4173 [a]	33084 ± 792 [b]
Ca	31092 ± 35708	10244 ± 2110	16593 ± 7069	23446 ± 11388	18535 ± 10116	39040 ± 17504
Carcass						
Cu	64.5 ± 3 [a]	70.3 ± 9.9 [ab]	62.4 ± 6.8 [a]	75 ± 10.8 [ab]	62.1 ± 14.11 [a]	86.1 ± 12.9 [b]
Zn	5102 ± 1056	6061 ± 422	6185 ± 1350	7543 ± 2969	4718 ± 142	5902 ± 256
Cd	1.4 ± 0.2 [a]	1.8 ± 0.58 [ab]	1.2 ± 0.2 [a]	2.18 ± 0.2 [b]	1.2 ± 0.4 [a]	2.4 ± 0.3 [b]
Na	262013 ± 9578 [ab]	216370 ± 36606 [b]	288288 ± 23827 [a]	220686 ± 46033 [b]	259297 ± 41791 [ab]	210607 ± 17574 [b]
K	293946 ± 10943 [ac]	276700 ± 18322 [cb]	305503 ± 18203 [ac]	262871 ± 6071 [b]	279691 ± 7656 [acb]	263002 ± 15195 [b]
Mg	77609 ± 2719	78302 ± 5427	81638 ± 8928	79433 ± 8573	77123 ± 9383	79060 ± 3819
Ca	1191245 ± 111059	1152318 ± 151146	1329737 ± 260389	1313185 ± 293420	1198442 ± 223324	1250524 ± 98351