Mixed metal and temperature stress in aquatic environments

Giovanni Castaldo

Mixed metal and temperature stress in aquatic environments: establishing functional links across different levels of organisation

Giovanni Castaldo

Promotoren **prof. dr. De Boeck Gudrun | prof. dr. Ronny Blust**

Proefschrift voorgedragen tot het behalen van de graad van doctor in de wetenschappen: biologie
Faculteit Wetenschappen | Departement Biologie | Antwerpen, 2021

Universiteit Antwerpen

Faculteit Wetenschappen

Departement Biologie

Mixed metal and temperature stress in aquatic environments: establishing functional links across different levels of organisation.

Gecombineerde metaal en temperatuurstress in het aquatisch milieu, functionele verbanden tussen effecten op verschillende niveaus van organisatie

Proefschrift voorgelegd tot het behalen van de graad van doctor in de wetenschappen: biologie aan de Universiteit Antwerpen te verdedigen door

Giovanni Castaldo

Promotoren:

Prof. Dr. Gudrun de Boeck

Prof. Dr. Ronny Blust

Antwerpen, 2021

Members of the Jury

Chairman
Prof. Dr. Lieven Bervoets, University of Antwerp, Belgium

Promotors
Prof. Dr. Gudrun De Boeck, University of Antwerp, Belgium
Prof. Dr. Ronny Blust, University of Antwerp, Belgium

Members
Prof. Dr. Martin Grosell, University of Miami, Florida
Prof. Dr. Christer Hogstrand, King's College London, UK
Prof. Dr. Dries Knapen, University of Antwerp, Belgium
Prof. Dr. Patrick Meire, University of Antwerp, Belgium

This project was funded by the TOP BOF project 32252 granted by the University of Antwerp Research Council.

Contact information

Giovanni Castaldo

University of Antwerp - Department of Biology

Groenenborgerlaan 171, B-2020 Antwerpen

Belgium
giovanni.castaldo@uantwerpen.be

Sphere
Systemic Physiological & Ecotoxicological Research
University of Antwerp

Acknowledgments

Undertaking a Ph.D. is an outstanding and life-changing experience and arriving at this stage would not have been possible without the help of numerous people, who I wish to warmly acknowledge.

First and foremost, I am deeply grateful to my promoters for giving me the opportunity to start and bring to the end this important chapter of my life. I would like to express my special thanks to Prof. Dr. Gudrun De Boeck for her valuable time and continuous support. Without her help and supervision this PhD would have not been possible. You always found some spare time to discuss problems, review papers and mostly important to encourage me.

I also own my sincere gratitude to Prof. Dr. Ronny Blust for giving me this chance and letting me be part of the SPHERE team.

Besides my supervisors, I would like to thank Prof. Dr. Lieven Bervoets and Prof. Dr. Raewyn M. Town for their help, time and feedback, which allowed me to broaden my knowledge.

Moreover I would like to thank all the jury members: Prof. Dr. Martin Grosell, Prof. Dr. Christer Hogstrand, Prof. Dr. Dries Knapen and Prof. Dr. Patrick Meire for their time and constructive criticisms, which helped me to widen my research perspectives.

Furthermore I acknowledge the help of the whole technical staff of the SPHERE group and a special thanks goes to Steven Joonsen, Karine Van den Bergh and Dr Valentine M. Kayawe.

I want to extend my sincere gratitude to my colleagues: Bart Slootmaekers, Marion Pillet, Camilla Catarci Carteny, Elvio Amato, Vyshal Delahaut, Cara Byns, Sophie Gresham, Ali Pilehvar, Bossissi Nkuba, Lies Teunen, Gunter Flipkens, Hanne Hetjens, Kristine De Schamphelaere, Julia Camacho Garcia, Robin Lasters and Thimo Groffen.

I would like to express my sincere thanks to Diana, who always encouraged me to go on. Without her support this path would have been more difficult to conclude.

Above all I would like to thank my parents Rosanna and Michele for believing in me and for sharing their strength with me. My lovely sister Maria Pia for encouraging me all the time. Without them, this accomplishment would not have been possible.

Table of Contents

Thesis abstract

Worldwide, aquatic ecosystems are under threat from metal pollution. However, even though in the past years more attention has been given to the mixture of pollutants, the environmental quality standards are still mainly based on classical laboratory tests using single compounds. Proper environmental management requires not only proper monitoring approaches, but also effective quality standards. Within this context initiatives to include mixture effects are being taken. Natural environments receive a wide variety of compounds and the prediction of mixture toxicity based on the toxicity of single compounds is difficult and shows a certain degree of uncertainty. Moreover, the effects of metal mixtures together with the effect of the temperature on toxicological processes remains very poorly documented. Considering that the temperature is one of the most important driving factors in organismal physiology and a crucial ecological factor, this is surprising.

Within this framework, the aim of the present doctoral project was firstly to evaluate the outcome of single metal exposure on common carp as model organism. In the first chapter common carp was exposed to similar toxicity levels of three metals, namely copper (Cu), zinc (Zn) and cadmium (Cd). Our results showed differences in the accumulation of these metals, with Cu and Cd accumulating to a greater extent compared to Zn. Moreover, Cu was the only metal found to cause an impairment in ion-homeostasis. Afterwards, in the remaining chapters of the thesis, common carp was exposed for one week to several binary metal mixtures of Cu/Cd and Cd/Zn, and later on to trinomial mixtures. The main aim was to evaluate the response of common carp to a mixture stress and to observe if metals showed additive, synergistic or antagonistic-like adverse effects in the mixture. In general only a limited inhibition of one metal (e.g Cd and Cu) on the uptake of the other metal (e.g Zn and Cd) were noticed. However synergistic-like adverse effects of Cu and Cd mixture (Cu_{fix}/Cd_{50}) on calcium (Ca) levels were observed. In fact none of the three metals was found to impair Ca-levels in the single metal exposure scenario. This lack of effect was related not only to the relatively short exposure period, but also the protective role played by water-hardness towards metal toxicity. Moreover, considering that all three metals stimulated the response of defensive mechanisms in terms of metallothionein (MT) gene induction, we assume that the metal ions were bound to these proteins. When Cu, Cd and Zn were present together at relatively low concentrations representing the 10% of the 96h-LC_{50}, the first two metals accumulated quite rapidly, whereas Zn levels, as expected, remained stable. Even though Cu levels might have been slightly increased due to the mixture effect, an inhibitory effect of Cu on Cd and Zn accumulation was shown. Moreover the presence of the three metals together led to a fast sodium (Na) drop in the gills, which was not observed in the single exposure scenario. Thus a synergistic-like adverse effects of these metals on ion-homeostasis cannot be excluded. Nevertheless the fish were able to cope with this situation, keeping the Na loss under control, at least for the duration of the experiment. As

mentioned above, few studies were conducted to better understand the role of the temperature and of metal mixtures on aquatic organisms. Within this context, the last chapter of the present thesis focuses on understanding to which extent, in a long term study (27 days), different temperatures can affect metal mixture toxicity in common carp. In fish exposed to either a low (10 °C) or high (20 °C) temperature, both Cu and Cd accumulated in the gills, whereas Zn levels remained stable. However in fish exposed at 10 °C, Cu metal levels in the gills were higher as compared in fish at 20 °C, in contrast to what observed for Cd. Moreover at 20 °C, after one week of exposure fish started to eliminate excess Cu, and Cu levels in the metal-treated group returned to levels similar to the control. In addition, the effect of temperature on the organisms became clear; in fact the condition factor and the hepatosomatic index were higher in fish exposed at 10 °C compared to fish exposed at 20 °C. This suggested that fish had a decreased standard metabolic rate, which in turn led to an increased energy availability for growth and energy storage. Finally in this last chapter, the role played by the food was highlighted. In fact, in contrast to all the previous chapters fish were fed because they were kept under the exposure conditions for 27 days. The presence of food seems to have masked the electrolyte loss (mainly Na) due to the metal exposure. It is clear that more studies designed to underline the effect of food are needed to further investigate this aspect in toxicological studies.

Overall the present thesis provides new insights in the context of multi-metal and multi-stressor scenarios. However, in order to better understand metal toxicity and develop better water quality guidelines, it is necessary to conduct more experiments on pollutant and stressor mixtures taking into account several parameters, such as food, pH and salinity. Moreover it is necessary to compare and validate the data obtained in the lab, with results obtained in more realistic, ecologically relevant scenarios in order to set appropriate water quality standards.

Nederlandstalige samenvatting

Wereldwijd worden aquatische ecosystemen bedreigd door metaalverontreiniging. Maar ook al is er de afgelopen jaren meer aandacht besteed aan mengsels van polluenten, toch zijn de milieukwaliteitsnormen nog voornamelijk gebaseerd op klassieke laboratoriumtesten met enkelvoudige componenten. Goed milieubeheer vereist niet alleen een goede monitoring, maar ook effectieve kwaliteitsnormen. In dit kader worden initiatieven genomen zoals het mee opnemen van mengseleffecten. Natuurlijke omgevingen ontvangen een grote verscheidenheid aan chemische verbindingen en het voorspellen van mengseltoxiciteit op basis van de toxiciteit van afzonderlijke verbindingen is moeilijk en vertoont een zekere mate van onzekerheid. Ook het effect van metaalmengsels in combinatie met het effect van temperatuur op toxicologische processen is zeer slecht gedocumenteerd. Dit is verrassend gezien het feit dat de temperatuur één van de belangrijkste drijvende factoren is in de fysiologie van organismen en een cruciale ecologische factor.

Binnen dit kader is het doel van dit doctoraatsproject om allereerst de uitkomst van enkelvoudige metaalblootstelling op karpers als modelorganisme te evalueren. In het eerste hoofdstuk werden karpers blootgesteld aan vergelijkbare toxiciteitsniveaus van drie metalen, namelijk koper (Cu), zink (Zn) en cadmium (Cd). Onze resultaten lieten verschillen zien in de accumulatie van deze metalen, waarbij Cu en Cd in sterkere mate accumuleren in vergelijking met Zn. Bovendien was Cu het enige metaal dat de ion-homeostase uit balans bracht. Daarna, in de resterende hoofdstukken van het proefschrift, werden karpers gedurende een week blootgesteld aan verschillende binaire metaalmengsels van Cu / Cd en Cd / Zn en aan trinomiale Cu /Cd / Zn mengsels. Het belangrijkste doel was om de reactie van de karpers op mengselstress te evalueren en om te observeren of metalen in het mengsel additieve, synergetische of antagonistische effecten vertoonden. In het algemeen werden beperkte antagonistisch-achtige effecten van één metaal (bijvoorbeeld Cd en Cu) op de opname van het andere metaal (bijvoorbeeld Zn en Cd) opgemerkt. Er werden ook synergetisch-achtige effecten van Cu en Cd mengsel (Cu_{fix}/Cd_{50}) op calcium (Ca) niveaus waargenomen. In feite bleek geen van de drie metalen in het scenario met één metaal de Ca-niveaus te verminderen. Dit gebrek aan verstoring hield niet alleen verband met de relatief korte blootstellingsperiode, maar ook met de beschermende rol van waterhardheid tegen metaaltoxiciteit. Gezien het feit dat alle drie de metalen de respons van verdedigingsmechanismen in termen van inductie van het metallothioneïne (MT) gen stimuleerden, nemen we aan dat de metaalionen aan deze eiwitten gebonden waren. Wanneer Cu, Cd en Zn samen aanwezig waren, in relatief lage concentraties die de 10% van de 96h-LC_{50} vertegenwoordigden, accumuleerden de eerste twee metalen vrij snel, terwijl de Zn-niveaus, zoals verwacht, stabiel bleven. Hoewel de Cu-niveaus mogelijk enigszins verhoogd waren door een mengseleffect, werd een inhiberend effect van Cu op de accumulatie van Cd en Zn aangetoond. Bovendien leidde de aanwezigheid van de drie metalen samen tot een snelle daling

van natrium (Na) in de kieuwen, een effect dat niet werd waargenomen in het scenario van de enkelvoudige blootstellingen, dus een synergetisch effect van deze metalen op de ion-homeostase kan niet worden uitgesloten. Desalniettemin was de vis in staat om met deze verstoring om te gaan en het Na-verlies onder controle te houden, althans voor de duur van het experiment. Zoals hierboven vermeld, zijn er weinig onderzoeken uitgevoerd om de rol van de temperatuur in combinatie met metaalmengsels op aquatische organismen beter te begrijpen. Binnen deze context richt het laatste hoofdstuk van dit proefschrift zich op het begrijpen van de mate in welke verschillende temperaturen de toxiciteit van metaalmengsels bij karpers kunnen beïnvloeden in een lange termijn studie (27 dagen). Bij vissen die werden blootgesteld aan een lage (10 °C) of hoge (20 °C) temperatuur, stapelden zowel Cu als Cd zich op in de kieuwen, terwijl het Zn-gehalte stabiel bleef. Bij vissen die werden blootgesteld aan 10 °C, waren de Cu-metaalgehaltes in de kieuwen, in tegenstelling tot wat waargenomen voor Cd, hoger dan bij vissen bij 20 °C. Bovendien begonnen vissen bij 20 °C na een week blootstelling de overtollige Cu te elimineren, waarbij de niveaus in de met metaal behandelde groep terugkeerden naar niveaus die vergelijkbaar waren met die in de controle. Ook werd in het laatste hoofdstuk het effect van de temperatuur zelf op de organismen opgemerkt, in feite waren de conditiefactor en de hepatosomatische index hoger bij vissen blootgesteld aan 10 °C vergeleken met vissen blootgesteld aan 20 °C. Dit suggereerde dat vissen bij 10 °C een lagere standaard stofwisseling hadden, wat op zijn beurt leidde tot een grotere beschikbaarheid van energie voor groei en energieopslag. Ten slotte werd in dit laatste hoofdstuk de rol van voedsel belicht. In tegenstelling tot de voorgaande hoofdstukken, werden de vissen gevoerd aangezien ze 27 dagen onder de blootstellingsomstandigheden werden gehouden. De aanwezigheid van voedsel lijkt het verlies aan elektrolyten (voornamelijk Na) als gevolg van de blootstelling aan metalen te hebben gecompenseerd. Desalniettemin zijn er meer studies nodig om het belang van voedsel in toxicologische studies te onderstrepen en om dit aspect verder te onderzoeken.

Over het geheel genomen biedt dit proefschrift nieuwe inzichten in de context van multi-metaal en multi-stressoren scenario's. Om de metaaltoxiciteit beter te begrijpen en betere waterkwaliteitsrichtlijnen te ontwikkelen, is het nodig om meer experimenten uit te voeren met mengsels van verontreinigende stoffen en stressoren, rekening houdend met verschillende variabelen, zoals voedsel, pH en zoutgehalte. Bovendien is het nodig om de gegevens die in het laboratorium zijn verkregen te vergelijken en te valideren met resultaten verkregen in meer realistische, ecologisch relevante scenario's om de juiste waterkwaliteitsnormen vast te stellen.

Chapter 1.

General Introduction

1.1. Metals in the aquatic environment

Over the past decades more and more attention has been drawn to the occurrence of metal pollution in aquatic systems. Metals are naturally present in the aquatic environment due to the slow leach from rock to water (Zhou et al. 2008). However anthropogenic activities such as mining, industry, agriculture and urbanization led to an increase of metals in natural environments. The increase in metal pollution and the presence of toxic metals is a major concern for human health and ecotoxicology (Domingos et al. 2015).

Aquatic organisms living in contaminated environments, can accumulate metals directly from the water through the gills or via food (Perera et al. 2015). Generally metals can be divided in two main groups, namely essential and non-essential. The metal ions belonging to the first group are micro-nutrients needed for the organisms such as copper (Cu), zinc (Zn), iron (Fe) and manganese (Mn), however they can pose a risk for the organism when present at concentrations too low or too high. The metals falling in the latter group, such as mercury (Hg), lead (Pb) and cadmium (Cd) have no known biological functions in vertebrates (Danabas et al. 2018). Both essential and non-essential metals can be considered a threat to the environment due to their potential toxicity, persistence and ability to accumulate in the aquatic organisms and food chain (Begum et al. 2005, Bervoets et al. 2009, Díaz-de-Alba et al. 2017). Metal uptake and toxicity are strongly affected by their bioavailability (Rainbow 2007). Dissolved free metal ions shows the fastest accumulation and uptake rates in organisms and are the most bioavailable form in aquatic environments (Luoma 1983, Sokolova and Lannig 2008).

In general both the essential and non-essential metals share some common potential toxicity effects such as the ability to induce oxidative stress and disturbance in ionoregulation (Alsop and Wood 2011, Sevcikova et al. 2011). Differences in the toxicities of metal ions might be related for instance to the chemical reactivity and speciation in the exposure media or to the exposure route. Nonetheless fish have several defensive mechanism to cope with increased metal levels in order to protect the organism from their deleterious effects.

In the present work three different metals, two of them essential (Cu and Zn) and one a non-essential element (Cd), were selected to evaluate their toxic effects when present alone or in a mixture at two different temperatures.

1.1.1. Copper

Copper, is an essential metal necessary for many fundamental biological processes such as cellular respiration, connective tissue formation and melanin production (Zhao et al. 2014). It is naturally present in the Earth's crust and can be found in surface waters of freshwater systems in a concentrations range from 0.003 to 0.47 µM (or 0.2 to 30 µg/l) (USEPA. 2007). However, due to anthropogenic input these concentrations can rise drastically (Grosell 2011). For instance, in Flemish (Belgium) rivers, concentrations of Cu up to 6.8 µM were reported by Bervoets et al. (2004).

Copper uptake in fish can occur directly via the gill epithelium, or as in higher vertebrates via diet (Grosell 2011). Gills can play an important role in Cu homeostasis, in fact under normal dietary circumstances, only ≃ 10% of the required Cu is taken up from the water via gills, however this percentage can increase above the 60 % in case the dietary source of Cu is reduced, suggesting that the uptake of Cu via this tissue, is somehow regulated on the organismal Cu status (Kamunde et al. 2002b). When the organism is exposed to increased dietary or waterborne Cu, the branchial metal uptake is reduced to protect the organism from deleterious effects (Grosell et al. 1997, Kamunde et al. 2002a, Grosell 2011).

In general, during waterborne Cu exposure, this metal is rapidly accumulated within the first hours of exposure, followed by a steady state with elevated and stable Cu levels (Grosell et al. 1997, Grosell et al. 1998). In freshwater fish gills (Fig. 1), Cu uptake occurs via transporters such as the divalent cation transporter (DMT1) and the high-affinity copper transporter (CTR1) (Bury et al. 2003, Komjarova and Bury 2014). Next to these two transporters, Cu can also be taken up via a putative apical sodium (Na^+) channel (Grosell and Wood 2002) coupled with the H^+-ATPase. Furthermore Cu uptake might be facilitated by exchangers such as the Na^+/H^+-exchangers (NHEs) (Grosell 2011, Komjarova and Bury 2014). It has been demonstrated that in rainbow trout (*Oncorhynchus mykiss*) copper uptake pathways can be divided into the Na^+-sensitive, which dominate at low Na^+ ambient levels and the Na^+-insensitive, which dominate in waters of high ionic strength (Grosell and Wood 2002). Two candidates were identified for the Na^+-insensitive pathways namely the CTR1 and DMT1, both of them expressed in fish gills. The former appears to transport the cuprous ion (Cu^+), whereas the DMT1 transport Cu^{2+} (Grosell 2011). Copper export is dependent on Cu-ATPase ATP7A and ATP7B (Grosell 2011, Zhao et al. 2014), which are expressed, respectively in proximity of the basolateral and apical membranes (Lutsenko 2010). In sea bream (*Sparus aurata*), the ATP7A in contrast to the ATP7B is expressed in the gill, thus it is a probable candidate for the basolateral Cu transport in fish (Minghetti et al. 2010).

Excesses of Cu can be excreted by the organism in different ways, however the mechanisms behind Cu excretion are not completely clear in fish (Minghetti et al. 2010, Grosell 2011). Copper can be excreted through the hepatobiliary system, the excretion

through this system is stimulated both by waterborne and dietary Cu exposure (Grosell et al. 1998, Kamunde et al. 2001). In addition Cu might also be excreted via urine, however it has been suggested that urinary Cu excretion is more important in marine teleosts rather than in freshwater organisms (Grosell 2011). Next to these two excretion pathways, excesses of Cu can be excreted through the gills, as demonstrated in rainbow trout treated with radiolabelled Cu (^{64}Cu) (Grosell et al. 2001).

When metal intake is not balanced with excretion, the accumulation of metal ions might result in toxic effects. For instance several studies reported detrimental neurological adverse effects, such as impairment of the olfactory abilities (Beyers and Farmer 2001, Green et al. 2010) or a reduction of learning abilities (Pilehvar et al. 2020). Furthermore overload of Cu can lead to production of reactive oxygen species (ROS), such as hydroxyl radicals, which can cause cellular damage leading to death (Pena et al. 1999, Zhao et al. 2014). Copper can also impact the immune system, for example short term Cu exposure at relatively low concentrations representing the 9% of the 96h-LC_{50} (concentration lethal for the 50% of the population in 96h) increased the mortality due to *Vibrio anguillarum* infections in the Chinook salmon (*Oncorhynchus tshawytscha*) (Baker et al. 1983). Furthermore Cu can also impair Na^+-homeostasis, due to the competition with this ion at the uptake site, as mentioned above, and inhibiting the activity of the sodium–potassium adenosine triphosphatase (Na^+/K^+-ATPase) (De Boeck et al. 2001, Mackenzie et al. 2004, Grosell 2011).

Exposure to Cu, shows as one of the most consistent adverse effects elevated plasma ammonia (Grosell et al. 2003, Grosell et al. 2004) and acid-base balance disturbance. For instance in rainbow trout, exposure to Cu and disruption of the gill epithelium have adverse effects on respiratory gas exchange, reducing haemoglobin oxygenation. This can lead to accumulation of carbon dioxide (CO_2), thus the fish switch to anaerobic metabolism causing an increase in lactic acid in the blood (Taylor et al. 1996).

1.1.2. Zinc

Zinc, is another essential element and is one of the most abundant trace metal in most vertebrates (Vallee et al. 1986). The concentrations of this metal in the environment show a great variation ranging from 0.30 nM (0.02 µg/l) in remote rivers to more than 30.5 µM (2000 µg/l) in areas near industries and mining activities (Bervoets and Blust 2003, Hogstrand 2011).

Similar to Cu, Zn also can be adsorbed by the intestine and the gills of fish (Bury et al. 2003). Under normal circumstances, Zn uptake occurs mainly through the gut, while the gills act as auxiliary organ. However, especially in freshwater fish, in case Zn concentrations in the water increase and the Zn supplied via food decrease, the uptake of this metal across the gills can rise significantly (> 50%) (Spry et al. 1988, Hogstrand 2011). Thus the gills are involved to supplement adsorption when required and the

intestine act as the bulk pathway for uptake (Bury et al. 2003). Zinc uptake (Fig. 1) starts with the binding of the cation to a negatively charged site located on the gill surface, followed by internalization (Pagenkopf 1983, Hogstrand and Wood 1996). Two main protein families are involved in Zn transport in animals, namely the Znt and Zip families (Zn transporter and Zrt irt-related protein respectively) (Hogstrand 2011). Under normal physiological conditions, the former family of proteins typically transport Zn away from the cytosol, whereas the latter family is involved in Zn transport into the cytosol. Moreover most of the Zip proteins, such as the Zip8 in fish gills, mediate the tissue specific Zn-uptake (Feeney et al. 2005, Hogstrand 2011). Next to the Znt and Zip proteins it has been demonstrated that calcium transporters are generally permeable to Zn (Bury et al. 2003). For instance Zn^{2+} can compete with calcium (Ca^{2+}) for the gills uptake via the epithelial calcium channel (ECaC) located on the apical membrane of gills (Hogstrand et al. 1994).

From a chemical perspective, Zn is not a direct antioxidant agent itself (Maret 2019), but when oxidants interacts with the thiolate causing the release of free zinc, a zinc signal that triggers an anti-oxidant response against oxidative stress occurs. Physiological Zn levels have pro-antioxidant properties, but deficiencies or excesses of this metal, can exert pro-oxidant effects and thus cause oxidative stress (Lee 2018, Maret 2019, Prasad and Bao 2019). Different mechanism have been proposed for the pro-antioxidant role of Zn: 1) proteins free sulfhydryl group protection; 2) outcompeting redox-active metals; 3) the stimulation of antioxidant system response (Lee 2018, Maret 2019). Moreover changes in free Zn concentrations are recognized by the metal-responsive transcription factor-1 (MTF-1) and once that Zn is bounded to the MTF-1 the induction of metallothionein (MT) expression occurs (Lee 2018).

As mentioned above, being one of the most abundant trace metal in the body, it is no surprise that Zn-homeostasis is tightly regulated. It was found that the uptake rate of Zn via gills, in the wild-caught yellow perch (*Perca flavescens*) from Zn-polluted rivers was decreased as compared with the same fish caught in a reference site (Niyogi et al. 2007). However this difference was only noticed in the gills and not in the gut, suggesting the importance of regulating Zn accumulation in the gills and indicating that Zn adsorption can be regulated independently in these two compartments (Niyogi et al. 2007). It has been shown that rainbow trout, when transferred to water with high Zn concentration (2.3 µM) can reduce the affinity (increased K_M) for Zn in the gills in order to slow down the uptake of this metal, suggesting a fast and selective decrease of the high-affinity Zn transporters (Hogstrand 2011).

Little is known about Zn excretion routes in fish, yet it can be quickly eliminated from gills, liver and kidney (Hogstrand 2011). Following a radioisotope experiment using ^{65}Zn, gills have been suggested as the major excretion route for Zn in rainbow trout (Hardy et al. 1987). In addition it appears that only $\simeq$ 1% of ^{65}Zn assimilated

trough diet or injection can be found in the bile (Hardy et al. 1987, Chowdhury et al. 2003). Similarly, the contribution of urine represent a small fraction (≃ 1%) in Zn excretion (Spry and Wood 1985, Hardy et al. 1987).

Also for Zn, when the intake is not balanced with excretion, the accumulation of metal ions might result in toxic effects. For instance, acute Zn exposure might cause metabolic acidosis via the stimulation of ammonia excretion/uptake (Spry and Wood 1985) and excessive Zn levels might cause oxidative stress (Zheng et al. 2016). In addition Zn is known to impair Ca^{2+} homeostasis through the competition with this ion at the uptake site or through the inhibition of the high affinity Ca^{2+}-ATPase, involved in the extrusion of Ca^{2+} across the basolateral membrane of gill epithelial cells (Hogstrand et al. 1996).

1.1.3. Cadmium

Cadmium is an ubiquitous element present at generally low concentrations in the geosphere (Thornton 1995). In the aquatic systems, Cd occurs generally at low concentrations. Usually in European freshwater and seawater the total dissolved Cd is respectively ≃ 0.004 µM and 0.18 nM (or 0.5 and 0.02 µg/l) (Pan et al. 2010). In addition, some field studies, reported Cd level up to ≃ 0.52 µM and ≃ 0.17 µM (Bervoets et al. 2005, De Jonge et al. 2014). According to the European directive 2008/105/EC, in Europe the maximum allowed concentrations for total dissolved Cd content in surface waters ranged between 0.004 to 0.013 µM according to the water hardness.

Cadmium uptake (Fig. 1) in fish occurs mainly via gills and intestine. It can be taken up via the skin and olfactory epithelium, however the uptake via the skin is relatively low compared to the one across the gills or the intestine (McGeer et al. 2011). Being a non-essential element in vertebrates, no specific Cd transporters are present in eukaryote cells. It has been demonstrated by several studies that Cd^{2+} act like a Ca^{2+} analogue, showing an antagonistic interaction between these two elements (Hollis et al. 2000a, Niyogi and Wood 2004, Niyogi et al. 2008, McGeer et al. 2011). Similar to Zn^{2+}, also the Cd^{2+} apical uptake is accepted to occur via the lanthanum-sensitive epithelial Ca^{2+}channel (Verbost et al. 1987, Verbost et al. 1989, Galvez et al. 2007). In addition to the uptake via the ECaC channel, Cd^{2+}can be taken up via the DMT1 (Bury and Grosell 2003, Cooper et al. 2007, McGeer et al. 2011). Moreover, in a recent study using zebrafish liver cell lines, an overexpression of the CTR1 increased Cd uptake, and this channel seems to be responsible for the transport of Cd into the cells (Kwok et al. 2020).

It is known that Cd^{2+} has a long half-life in vertebrates reflecting that no efficient excretion pathways are present in vertebrates for this non-essential element (McGeer et al. 2011). During dietary exposures, the gastrointestinal tract acts as a barrier to

prevent the metal absorption allowing the excretion of an important portion of ingested Cd via mucosal sloughing and via faeces (Chowdhury et al. 2004, McGeer et al. 2011). Moreover it has been demonstrated that due to acclimation to dietary exposure, the rate of Cd urinary excretion dramatically increases (Chowdhury and Wood 2007). Furthermore, chronic Cd waterborne and dietary exposures resulted in increased metal excretion rates via the hepatobiliary system (Chowdhury et al. 2003, Chowdhury et al. 2004, McGeer et al. 2011). The gills also play a role in excreting a small portion of accumulated Cd via the mucosal sloughing (Handy 1996, McGeer et al. 2011).

Toxic effects associated with waterborne Cd in freshwater fish are generally related with disturbance in Ca^{2+} homeostasis, which might results in hypocalcaemia (Cinier et al. 1997, McGeer et al. 2011). Moreover this metal is known to modulate protein structure and generate oxidative stress (Casalino et al. 2002, Matović et al. 2010, Ferain et al. 2018). For instance, this metal can increase the amount of free available Fe by replacing it in various proteins, such as ferritin and apoferritin, increasing the amount of freely available Fe ions that can generate ROS trough the Fenton reaction (Wätjen and Beyersmann 2004). Furthermore it has the ability to interfere with the electron transport chain resulting in an increased ROS production (Livingstone 2001) and to disturb the antioxidant system (Stohs and Bagchi 1995). Cadmium toxicity also shows adverse effects on growth and reproduction system (Lizardo-Daudt and Kennedy 2008, McGeer et al. 2011). In addition, it can lead to behavioural changes, such as decreased activity (Eissa et al. 2010) or impaired antipredator and exploration behaviours (Honda et al. 2008).

1.1.4. Metal interactions

As already mentioned above, ecosystems are often contaminated by a mixture of pollutants. Thus when multiple stressors are present, they might show additive, synergistic or antagonistic effects (Preston et al. 2000). The additive effect occur when the toxicity of multiple stressors is similar to the sum of the toxicity of each stressor taken individually. When the toxicity of a mixture is greater than or less than the sum of the toxicity of different stressor the interaction might be defined as respectively synergistic or antagonistic (Preston et al. 2000, Crain et al. 2008). Different metals can interact with each other influencing the uptake, bioaccumulation and toxicity (Komjarova and Blust 2009).

When chemicals are present as a mixture, they can interact with their respective uptake mechanisms and this can lead either to a stimulation or to an inhibition of the uptake of a particular compound. Moreover the type of interaction might be influenced by several factors, such as the metals involved, their ambient concentrations, studied species and exposure duration (Amiard-Triquet and Amiard 1998, Norwood et al. 2003).

In fish gills exposed simultaneously to Cu and Cd, an inhibition of Cu on Cd uptake has been observed, whereas no effects of Cd on Cu uptake where observed (Pelgrom et al. 1995, Komjarova and Blust 2009). Nevertheless Cd uptake after prolonged exposure to sublethal Cu levels seems to be enhanced (McGeer et al. 2007, Grosell 2011). In case of simultaneous exposure to Cu and lead (Pb), a synergistic-like effect on the uptake of both metals was shown in fish gills (Tao et al. 1999, Komjarova and Blust 2009). A non-competitive interaction on metal gill-binding was reported between Cu and silver (Ag) in rainbow trout (Niyogi et al. 2015). Zinc can compete with uptake mechanisms when present together with other metals such as Ni or Cd. For example Cd^{2+} and Zn^{2+} can compete with each other for uptake. This is explained by the chemical similarity between these two metals and their strong bond to the sulfhydryl groups (-SH) (Brzóska and Moniuszko-Jakoniuk 2001), which is stronger for Cd rather than for Zn. Thus a good binding site for Zn is considered to be an excellent binding site for Cd (Hogstrand 2011). Zinc has been shown to reduce Ni uptake, however considering that Ni does not interfere with ions such as Ca^{2+}, the mechanism behind this interaction is still uncertain (Komjarova and Blust 2009). In zebrafish, Cd uptake was reduced in presence of Zn, and the influence of Zn on Cd uptake and influx processes indicates that the influx of Zn and Cd occurs through common pathways (Glynn 2001). Cadmium is known to inhibit the Pb-gill binding in fish (Birceanu et al. 2008). This inhibition is likely to happen because Cd outcompetes Pb for the gill binding sites, which are likely the apical Ca^{2+}-channels (Rogers and Wood 2004, Rogers et al. 2005). Nevertheless the combination between these two metals shows more than additive adverse effects, aggravating the uptake of calcium and sodium (McGeer et al. 2011).

Considering that multi-metal interactions have been reported in several aquatic organisms under environmentally relevant concentrations (Rainbow et al. 2000, Birceanu et al. 2008, Komjarova and Blust 2008) it is necessary to take them into account when setting specific water quality criteria (Norwood et al. 2003).

1.2. Ionoregulation

Compared to animals living in the terrestrial environment, fish have to deal with more challenging ionic and osmotic gradients from aquatic environments. Thus, teleost have highly efficient ionoregulation mechanisms to regulate body fluids homeostasis (Hwang and Lee 2007). Ion- and osmoregulation in freshwater fish involves several processes. It is known that freshwater fish drink very little, moreover in order to balance the passive water uptake, they excrete large volumes of diluted urine, while actively adsorbing ions from the environment through the gills (Evans et al. 2005, Hwang and Lee 2007). Fish use specialized cells, called ionocytes, present in the gills or on the skin to compensate for the ion loss (Dymowska et al. 2012). In fish gills, the mitochondria-rich cells (MRCs), previously called chloride cells, are ionocytes responsible for the active transport of ions (Hwang 2009). Thus, their principal role in

the freshwater fish branchial epithelium is the uptake of ions e.g. Na^+, chloride (Cl^-) and Ca^{2+} from the surrounding water (Metz et al. 2003). Sodium uptake in fish gills (Fig. 1) has been proposed to occur via a putative apical Na^+-channel coupled with the proton-ATPase (H^+-ATPase), which gives the electromotive force for the uptake of this ion, and via the Na^+/H^+ exchangers (NHEs) (Lin and Randall 1993, McCormick 2001, Hwang et al. 2011). In common carp, it has been shown that the presence of an inhibitor (bafilomycin A) of the V-ATPase H^+ pump reduced the whole Na^+ influx in young common carp by the 70 %, suggesting that the motive force created by the H^+ pump plays an important role in the uptake of Na^+ (Fenwick et al. 1999). Moreover, in common carp, Cu exposure can lead to acidosis and higher CO_2 levels (De Boeck et al. 2007). Therefore protons can arise from intracellular CO_2 hydration (Grosell et al. 2007) and a higher proton pumping activity can occur to compensate the pH imbalance.

For the sake of clarity, in the present thesis when describing our results for the H^+-ATPase we refer to the V type H^+-ATPase subunit B. In freshwater gills the V type H^+-ATPase is believed to facilitate both the acid excretion and the Na^+ uptake (Perry et al. 2000). Similarly when we refer to the Na^+/K^+-ATPase we mean the α-subunit, which contains the binding sites for Na^+, K^+, and ATP. Thus has the major catalytic and ion transporting capacity of the enzyme (Nilsen et al. 2010).

The carbonic anhydrase (CA), is known to play a role both in the ion uptake and in the acid base regulation, by providing acid-base equivalents for the exchange with the environment (Hwang et al. 2011). For instance at the gill site, the CA catalyses the conversion of the CO_2 to H^+ and bicarbonate (HCO_3^-) for export to water (Gilmour and Perry 2009). Moreover by the hydration of CO_2, the CA provides ions for the exchange of Na^+ and Cl^- (Grosell 2011).

The Na^+ export from the gills to the extracellular fluids is mediated by the basolateral Na^+-K^+-ATPase (Perry 1997, Evans et al. 2005, Hwang et al. 2011). This Na^+ pump utilize the energy obtained from the hydrolysis of ATP to export 3 Na^+ from the cell to the extracellular fluids and to import 2 K^+ into the cell, contributing in to the resting membrane potential of animal cells (Handy et al. 2002).

Divalent ion concentrations, such as Ca^{2+}, magnesium (Mg^{2+}) and Zn^{2+}, in freshwater are below the plasma levels, fish must extract them from food or water (Evans et al. 2005). In contrast to terrestrial vertebrates, teleost bones are acellular, thus they do not provide a mobilizable Ca^{2+} pool (Liem et al. 2001, Evans et al. 2005). Calcium uptake occurs mainly via the ECaC located on the apical surface of the MRCs and it is extruded via the Ca^{2+}-ATPase (PMCA) and Na^+/ Ca^{2+} exchanger (NCE) located on the basolateral side of the membrane (Evans et al. 2005, Dymowska et al. 2012).

1.2.1. Metal and electrolyte levels

As mentioned in the previous sections, metals might compete with other ions present in the water (e.g Na^+ and $Ca2^+$) for the uptake via shared transport routes.

Copper is perhaps one of the most studied metal and its adverse effects on Na^+-homeostasis have been reported by several authors in different species (Grosell et al. 2002, De Boeck et al. 2010b, Niyogi et al. 2015). Copper might interfere with the apical Na^+ uptake into the cells in different ways. The interaction between the metal and a putative Na^+-channel is one of the reasons behind the competitive inhibition of Na^+ uptake. Nevertheless also the NHEs (e.g. NHE-2 and NHE-3) might be a target (directly or indirectly, via the inhibition of the CA leading to a substrate depletion needed for Na^+ uptake) for Cu-induced inhibition of Na^+ uptake (Grosell 2011). As already mentioned, the Na^+ export from the cell, is mediated by the basolateral Na^+/K^+- ATPase and, even though there is no evidence of Cu leaks through this pump, the inhibitory adverse effects of this metal on the Na^+-pump are known (Li et al. 1996, De Boeck et al. 2001, Handy et al. 2002).

Zinc is known to interfere with the Ca^{2+} homeostasis. This metal can compete for the uptake with Ca^{2+}, inhibiting the transfer of this ion across the apical membrane of the gill epithelial cells (Hogstrand et al. 1995). The uptake of Zinc occurs via the ZIP family (e.g. Zip1, Zip3 and Zip10), and the expression of some transporters involved in Zn uptake such as Zip10 can be regulated in case of Zn depletion or supplementation (Hogstrand 2011). Moreover, Zn uptake is believed to occur through the ECaC, which is permeable both to Zn^{2+} and Ca^{2+} (Shahsavarani et al. 2006, Hogstrand 2011). However, also the Ca^{2+}-ATPase, involved in the extrusion of Ca^{2+} might be a target of Zn^{2+} toxicity (Hogstrand et al. 1996).

The ability of Cd to interfere with ionoregulation has been observed repeatedly in several species such as the flounder (*Platichthys flesus* L.), the Atlantic salmon (*Salmo salar*) and rainbow trout (Larsson et al. 1981, Rombough and Garside 1984, McGeer et al. 2000b). Ionoregulation disturbance shows the classic damage-repair-acclimation scenario (McDonald and Wood 1993). For instance the disruption of branchial Ca, Na, K and Mg balance occur approximately within the first week of exposure and then the ion levels stabilize returning to control levels (McGeer et al. 2000b, Kalay 2006, McGeer et al. 2011). This trend with repair and recovery processes involves the production and mobilization of metal bindings molecules, such as metallothionein (MT) and glutathione (GSH), responsible for the metal sequestration and detoxification (Chowdhury et al. 2005, McGeer et al. 2011) and on the other hand the release of cortisol, elevated prolactin levels and increased gills chloride cells density (Fu et al. 1989, Fu et al. 1990). Moreover, Cd has the ability to replace Zn from the MT increasing

the amount of free Zn. Thus, Zn can interact with the MTF-1 stimulating the production of extra MT (Chung et al. 2005).

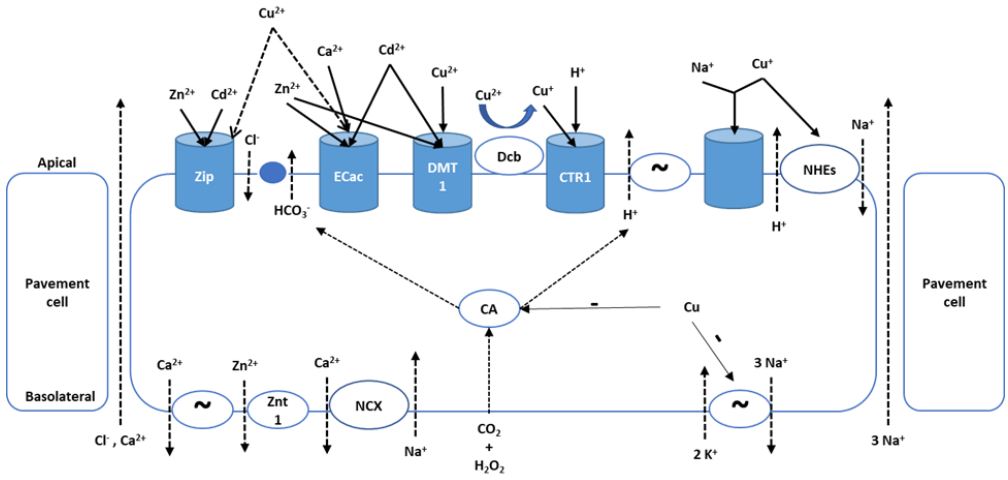


Figure 1: Schematic representation of suggested Cu, Zn and Cd uptake in freshwater fish gills using information derived from literature (Grosell 2011, Hogstrand 2011, McGeer et al. 2011, Komjarova and Bury 2014). In the figures are represented the putative apical Na^+ channel, the ATPase pumps (~), the zinc importers (e.g Zip10), the epithelial calcium channel (ECaC), the divalent metal ion transporter (DMT1), the Cu reductase (Dcb), the high affinity Cu transporter (CTR1), the Na^+/Ca^{2+}-exchanger (NCX), the Na^+/H^+-exchangers (NHEs) and the carbonic anhydrase (CA).

1.3. Defensive mechanisms

As mentioned in the previous paragraphs, metals have the ability to induce oxidative stress and cause extensive damages to the organism. Therefore fish have several defensive mechanisms to cope with metal toxicity.

Metallothioneins, are metal-binding proteins, cysteine-rich (33%) and with a low molecular weight (6000-7000 Da), which are known to play a key role in essential metal homeostasis and in sequestration and detoxification of non-essential metals (Hermesz et al. 2001, De Boeck et al. 2003, Reynders et al. 2006b, Atli and Canli 2008). Metallothioneins are formed of two globular domains, the α and the β-domain which contain eleven and nine cysteine residues respectively (Rhee et al. 2009, Vergani 2009). Moreover, the α-domain can bind up to four bivalent metal ions, whereas the β domain can bind up to three bivalent metal ions (Dziegiel et al. 2016). These proteins have a different binding strength for different metals following the order $Co^{2+} < Zn^{2+} < Pb^{2+} < Cd^{2+} < Ag^{+}$, $Cu^{+} < Hg^{2+}$ (Vašák 1991).

In fish, metal exposure results in the induction of MT production. In common carp and in crucian carp (*Carassius cuvieri*), the administration of metal ions is known to induce the production of two MTs (Muto et al. 1999, Ren et al. 2000, Chan et al. 2004). In control common carp the MT-1 gene is expressed at similar levels between various tissues (e.g. liver, muscle, brain kidney). Moreover the amount of MT-1 mRNA in all the analysed tissues (with the exception of the heart) was higher compared to the MT-2 mRNA levels (Hermesz et al. 2001). However during metal exposure, the MT-2 is more induced compared to the MT-1 (Hermesz et al. 2001). In adult male zebrafish exposed to Cd^{2+}, a strong induction of MT-1 occurred in the liver and in the gills, suggesting that this isoform could play a significant role in metal detoxification (Bourdineaud et al. 2006). Moreover, in a study done on common carp by Hermesz et al. (2002) it was noticed that Cu stimulated the response of both the MT-1 and MT-2 in the liver of common carp. Next to the MTs, fish have various antioxidant systems necessary to protect the organism from metal-related oxidative stress (Atli and Canli 2007, Eroglu et al. 2015). Antioxidant defences comprise both enzymatic and non–enzymatic antioxidants. The most important non-enzymatic antioxidants are the reduced glutathione (GSH), ascorbic acid also known as vitamin C, retinol or vitamin A and α-tocopherol known as vitamin E (Livingstone 2001). The enzymatic antioxidants, involved in the metabolism of the glutathione and/or in the removal of ROS are the glutathione reductase (GR), glutathione S-transferase (GST), glutathione peroxidase (GPX), catalase (CAT), and superoxide dismutase (SOD) (Livingstone 2001, Pinto et al. 2003, Tripathi et al. 2006). The transcription of antioxidant genes, such as the GST, is activated depending on the nuclear factor erythroid 2-related factor 2/antioxidant responsive element (Nrf2/ARE) signal pathway (Osburn and Kensler 2008, Zhu et al. 2018b, Prasad and Bao 2019).

Glutathione, together with the MTs, represent an important line of defence as chelating agent for metals. It is a non-protein thiol involved in cellular defence against xenobiotics and ROS (Meister and Anderson 1983, Lange et al. 2002), it can alter metal uptake and elimination rates (Kang and Eenger 1987, Ochi et al. 1988). GST plays a role in the oxidation of GSH, enhancing the conjugation of compounds for a subsequent excretion (Larsson et al. 2002). The GSH oxidation may also scavenge free radicals, stabilize xenobiotics in their oxidative state and block lipid peroxidation (Dautremepuits et al. 2009). The oxidized form of the glutathione (GSSG) is converted in GSH by a reaction catalysed by the GR. Thus this enzyme plays a key role in maintaining a constant ratio GSH/GSSG (Dautremepuits et al. 2009). The SOD represent the first line of defence against ROS, it catalyse the formation of hydrogen peroxide (H_2O_2) from the superoxide radical ($O_2^{\bullet-}$). The newly formed H_2O_2 is further converted by the reactions catalysed by the CAT and GPx into water (H_2O) and oxygen (O_2) (Livingstone 2001, Pillet et al. 2019) (Fig. 2).

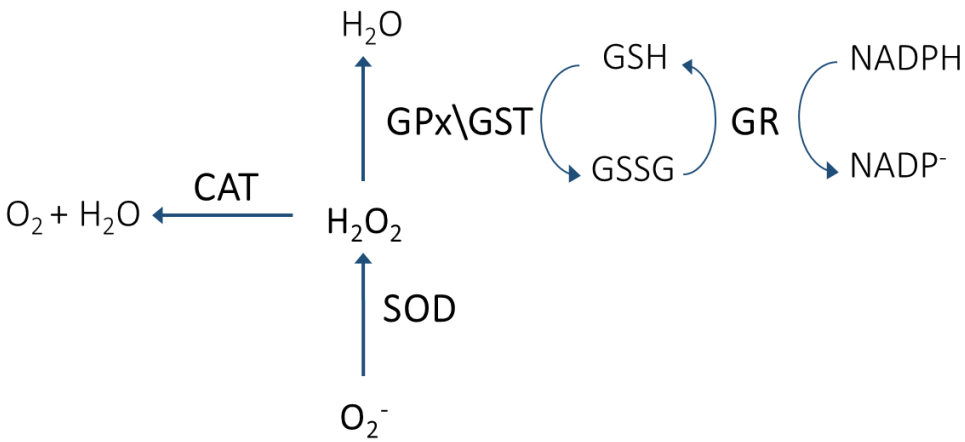


Figure 2: General representation of antioxidant system adapted form Weydert and Cullen (2010) and Hasanuzzaman et al. (2017).

1.4. Temperature

Temperature, impacting all the levels of biological organization is a key determinant of living organisms performance. Organisms can be divided into two main groups, ectotherm and endotherms. Organisms belonging to the former group have a body temperature similar to the one of their habitat, whereas the second group of organisms can generate heat and regulate the body temperature (Guderley 2004). The majority of organisms living in the aquatic systems are ectotherms (Willmer et al. 2009). Water breathing organisms, such as fish, are obligatory ectotherms, as the water current generated by the organism to ensure sufficient oxygen uptake at the gills also drains the heat that is produced due to its high specific heat capacity. Full oxygenation of blood in the gills requires a transit time, which is too long for a thermal gradient to be maintained between the water and the blood (Guderley 2004). Thus, the environmental temperature plays a key role in the physiology of these organisms (Hochachka and Somero 2002), and their use for the study of metabolic processes according to temperature changes can be considered as particularly advantageous (Guderley 2004).

Fish, according to their thermal range can be divided in stenotherm, with a narrow temperature range, and eurytherm, that tolerate a wide thermal range (Guderley 2004). Changes in water temperature can occur as a consequence of anthropogenic activities or naturally, e.g. due to the day/night cycle or seasonal changes. Ambient temperature changes can affect fish biology at different levels. For instance at cellular level, the temperature can influence the properties of biomolecules, metabolite levels and the cells structural components (e.g. proteins stability, lipids rigidity and permeability of the membrane) (De Boeck et al. 1996, Long et al. 2012). At physiological level it can affect the metabolism, plasma osmolarity and ionoregulation (Caulton 1978, Metz et al. 2003). Furthermore the temperature has effects also at population levels by affecting the fish growth and reproduction (Handeland et al. 2008, Dolomatov et al. 2013). Nevertheless, fish can respond to temperature alterations physiologically, via thermal adaptation and via behavioural thermoregulation (Ohlberger et al. 2008, Ward et al. 2010).

The role of the temperature on metal toxicity has been studied extensively by different authors (Guinot et al. 2012, Vergauwen et al. 2013, Pereira et al. 2017). Generally, it can be proposed that metal toxicity increase with the increases of the temperature. For example, the elevated metal toxicity at higher temperatures can be related to an increased uptake rate and thus to higher metal levels in the organism. Additionally, elevated metabolic rates result in higher energy demands, and as consequence in elevated ventilation and feeding rates leading to higher exposure to water and food contaminated by metals (Sokolova and Lannig 2008). However, this is not always the case, in fact at higher temperatures, also depuration processes might be facilitated

(Carvalho et al. 2004, Carvalho and Fernandes 2006). Moreover earlier studies reported no or reduced effects of increasing temperature on metal bioaccumulation and toxicity (Lannig et al. 2006, Pereira et al. 2017).

According to the review by Sokolova and Lannig (2008) (Fig. 3), five different toxicant-temperature patterns, depending both on the species specific optimal temperature and temperature range have been proposed. Of these patterns, the type I and II, both showing an increased toxicity with an increasing temperature are the most common for metals. The type III, which is more common for organic pollutants, shows a decreasing toxicity with increasing temperature. The type IV (optimum response) can be found in mammals with a minimal pollutant toxicity in the thermoneutral zone of endothermic animals. In the type V, the pollutant toxicity does not change according to the temperature and is rarely observed in aquatic ectotherms.

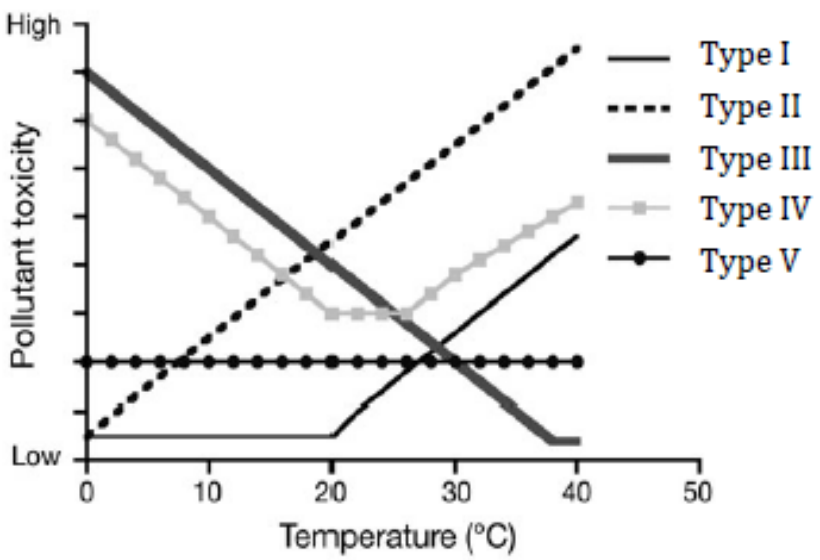


Figure 3: General representation of pollutant toxicity patterns as function of ambient temperature in aquatic ectotherms from the review of Sokolova and Lannig (2008).

1.5. Aim and outline of the thesis

The structural and functional organization of both aquatic and terrestrial ecosystems, as well as the services they can provide are threatened by physical and chemical pollution (Hering et al. 2010). Aquatic environments, being the final sink and receptors of waste streams are particularly at risk (Schwarzenbach et al. 2006). In order to protect the ecosystems various initiatives have been started (e.g. The Water Framework Directive and the REACH) and environmental quality standards (EQS) have been set. However, most of the tests to derive EQS have been performed on single compounds, thus the prediction of mixture toxicity is hard with a considerable uncertainty (Altenburger et al. 2004). **Therefore the aim of the present work, is to provide a better understanding and new insights for metal accumulation and toxicity in multi-metal exposure scenarios. Furthermore the interaction of metal mixtures with an environmental stressor such as the temperature has been assessed.**

In **Chapter 1**, a general introduction about metals in the aquatic environment, their toxic effects, defensive mechanisms of aquatic organism and the effect of temperature on metal toxicity has been given. In addition, a general overview of metal speciation under nominal metal concentrations used in this thesis can be found in table 1.

Table 1: Chemical speciation in the exposure media, calculated using nominal metal concentrations, at pH 7.8 with the equilibrium speciation code VMinteq.

Metal	Nominal metal concentrations µM	Free metal ion concentrations µM	% of total concentrations
10% LC_{50} Cu^{2+}	0.08	0.005	6.85
25% LC_{50} Cu^{2+}	0.19	0.013	6.85
50% LC_{50} Cu^{2+}	0.38	0.026	6.85
100% LC_{50} Cu^{2+}	0.77	0.053	6.85
10% LC_{50} Zn^{2+}	2.98	2.286	76.47
25% LC_{50} Zn^{2+}	7.47	5.715	76.48
50% LC_{50} Zn^{2+}	14.95	11.432	76.49
100% LC_{50} Zn^{2+}	29.89	22.874	76.53
10% LC_{50} Cd^{2+}	0.02	0.017	84.98
25% LC_{50} Cd^{2+}	0.05	0.042	84.98
50% LC_{50} Cd^{2+}	0.10	0.085	84.98
100% LC_{50} Cd^{2+}	0.20	0.170	84.98

In **Chapter 2** three parallel short term exposures (one week) using individually Cu, Zn and Cd have been performed on common carp (*Cyprinus carpio*). The nominal waterborne metal levels used corresponded to the 25%, 50% and 100% of the 96h-LC_{50} concentrations previously determined in our lab (Delahaut et al. 2020). This

experiment was done in order to understand the toxic effects of each individual pollutants in terms of fish survival rate, metal bioaccumulation, ionoregulation and defensive response. In **Chapter 3**, common carp were exposed for one week to several metal mixtures of Cu plus Cd in two experimental series. Each experimental series, consisted of a fixed concentration of one of the metals at 25 % of its 96h-LC_{50} combined with 10, 25 and 50 % of the 96h-LC_{50} of the other metal. The main aim was to evaluate the interaction between these two metals, their bioaccumulation, negative effects on electrolyte levels and responses of defensive mechanisms. Similarly, in **Chapter 4** the same endpoints were investigated using several metal mixtures of Cd and Zn. In **Chapters 5** and **6**, common carp was exposed to a ternary mixture of Cu, Zn and Cd at a concentration representing the 10% of the 96h-LC_{50} for each metal in order to assess the adverse effects on the endpoints mentioned above. The experiment in chapter 5 was a short term exposure (one week) at a fixed temperature of 20 °C with starved fish. The experiment in chapter 6 lasted for 27 days, and the aim was to understand to which extent different temperatures can affect metal mixture toxicity in common carp. Thus the fish were kept at two different temperatures representing relevant winter and summer temperatures in common carp's habitats (10 °C and 20 °C) and the fish were fed for the whole experiment. Finally in the last chapter of this thesis **(Chapter 7)** a general discussion of the results and findings obtained in the previous chapters has been given. In addition in this final chapter also the findings obtained in two extra works (Pillet et al. 2019, Pillet et al. 2020) strictly related with the aim of this thesis will be included. Furthermore this thesis will be concluded with suggestions for future studies in this field and perspectives for environmental risk assessment.

Finally as already mentioned above the general aim of the present thesis is to compare the adverse effects of metals in single and multi-stressors scenarios in order to have a better understanding of the complexity of metal interactions and their adverse effects when present together. Moreover, in general the metal concentrations used in the present experiments can be defined as environmentally relevant. This is important for future research that aims to compare results obtained in the a controlled environment (e.g. laboratory exposures) with results obtained in more-realistic scenarios (e.g. mesocosm) and/or directly in the field.

Chapter 2.

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

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

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

Modified version of Journal of Applied Toxicology (2020); pp 1–14

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 LC_{50} (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 amounts 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.

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

2.1. 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 thousands of proteins, and is a key player in cellular homeostasis, oxidative stress, aging, and immune response, amongst others (Fırat et al. 2009, Zhao et al. 2014). Moreover non-toxic Zn levels have pro-antioxidant properties and changes in free Zn concentrations are recognized by the metal-responsive transcription factor-1 (MTF-1). Once that Zn is bounded to the MTF-1 the induction of metallothionein (MT) expression occurs (Lee 2018). 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 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 LC_{50}. 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.

2.2. Material and Methods

2.2.1. 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).

2.2.2. 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.

2.2.3. 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. (2006a) 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).

2.2.4. 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.

2.2.5. 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.

2.3. Results

Measured exposure concentrations taken from every experimental day (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 LC_{50}) 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 LC_{50}) which showed on average values which were 10% below the intended exposure level.

2.3.1. 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.

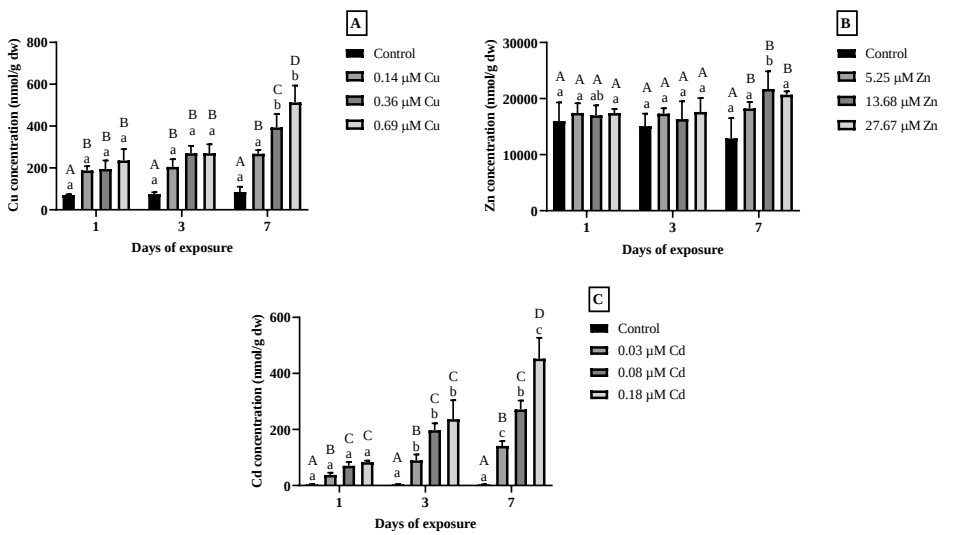


Figure 1: (A) Cu, (B) Zn, (C) Cd accumulation in the gills (nmol/g dw) of *Cyprinus carpio* exposed to different metals concentrations for 1, 3 and 7 days. Capital letters indicate significant differences between treatments during the same sampling day ($p < 0.05$). Lowercase letters indicate significant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

2.3.2. Defensive mechanisms

2.3.2.1. Metallothionein gene expression

2.3.2.1.1. 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.

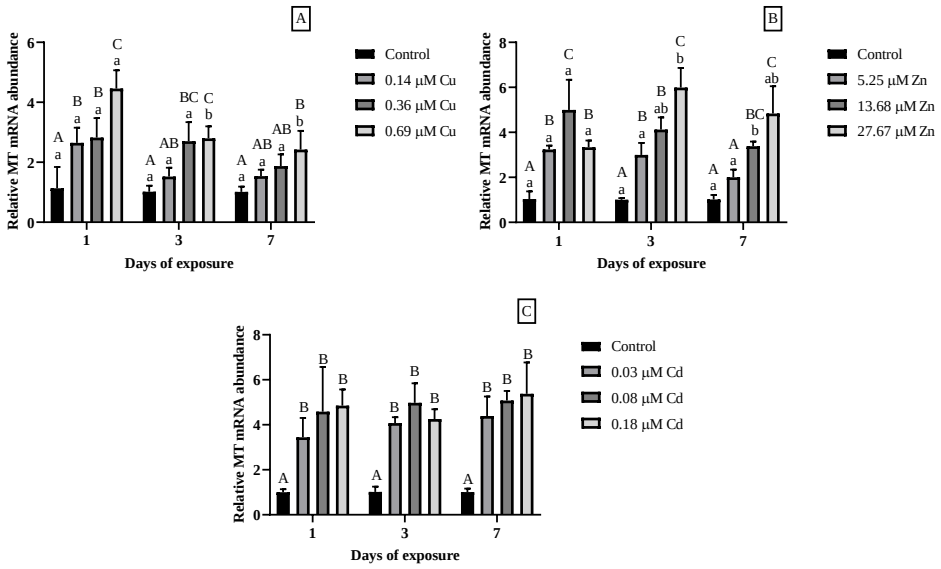


Figure 2: Relative MT mRNA abundance in *Cyprinus carpio* gills of fish exposed to different concentrations of (A) Cu, (B) Zn and (C) Cd, for 1, 3 and 7 days (mean ± SD) (N=4). Capital letters indicate significant differences between treatments during the same sampling day ($p < 0.05$). Lowercase letters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

2.3.2.1.2. 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.

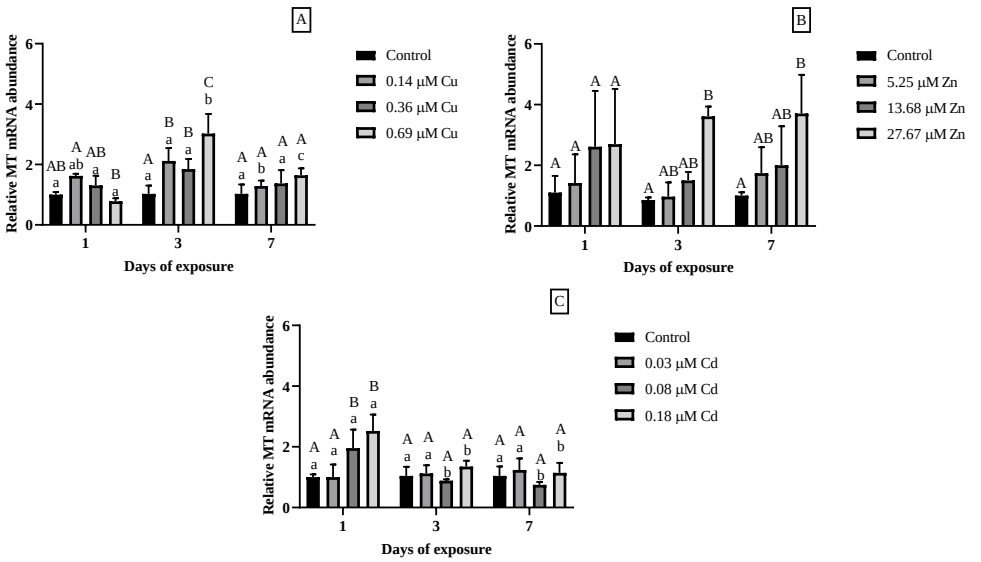


Figure 3*:* Relative MT mRNA abundance in *Cyprinus carpio* liver of fish exposed to different concentrations of (A) Cu, (B) Zn and (C) Cd, for 1, 3 and 7 days (mean ± SD) (N=4). Capital letters indicate significant differences between treatments during the same sampling day ($p < 0.05$). Lowercase letters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

2.3.2.2. 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).

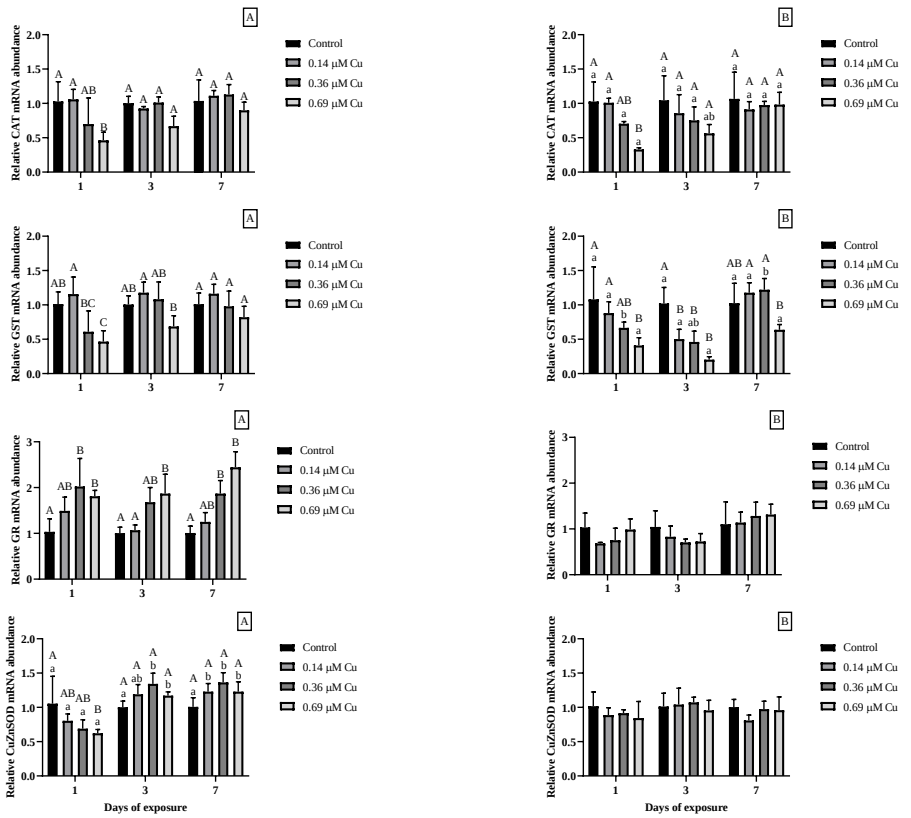


Figure 4: Relative CAT, GST, GR and CuZnSOD mRNA abundance in *Cyprinus carpio* (A) gills and (B) liver exposed to different Cu concentrations for 1, 3 and 7 days (mean ± SD) (N=4). Capital letters indicate significant differences between treatments during the same sampling day ($p < 0.05$). Lowercase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

2.3.3. 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).

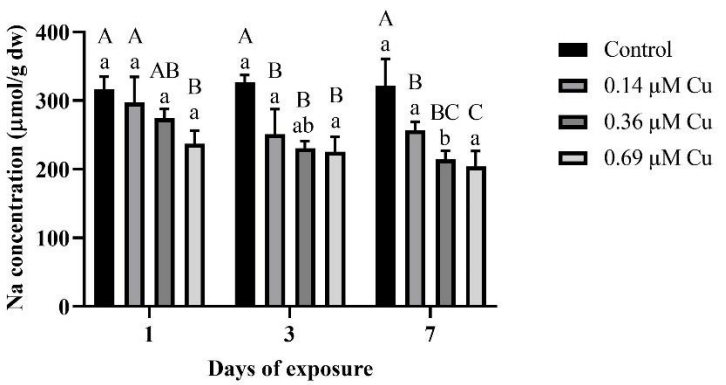


Figure 5: Na levels (µmol/g dw) in the gills of *Cyprinus carpio* exposed to different concentrations of Cu for 1, 3 and 7 days (mean ± SD) (N=5). Capital letters indicate significant differences between treatments during the same sampling day ($p < 0.05$). Lowercase letters indicate significant differences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

2.3.4. 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).

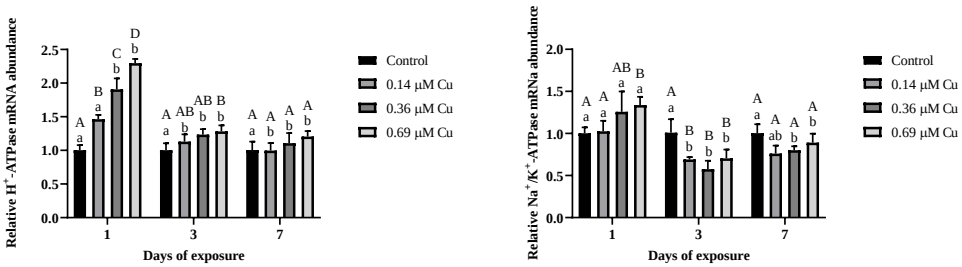


Figure 6: Relative H^+-ATPase and Na^+/K^+-ATPase mRNA abundance in *Cyprinus carpio* gills exposed to different Cu concentrations for 1, 3 and 7 days (mean ± SD) (N=4). Capital letters indicate significant differences between treatments during the same sampling day ($p < 0.05$). Lowercase letters indicate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

2.4. 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-LC_{50}. 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).

2.4.1. 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, 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 LC_{50} 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 LC_{50} 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. (2000a) 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.

2.4.2. 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. 2008b). 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.

2.4.3. 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 and in the neotropical *Prochilodus scrofa* (De Boeck et al. 2001, Cerqueira and Fernandes 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. 2010b). Thus the low mortality in the present experiment, in addition with the lower intended metal concentrations in the media and the LC_{50} 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. 2008b). 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 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.

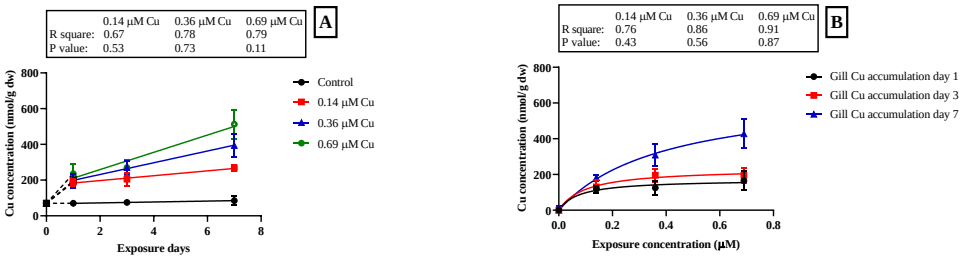
2.5. Conclusion

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.

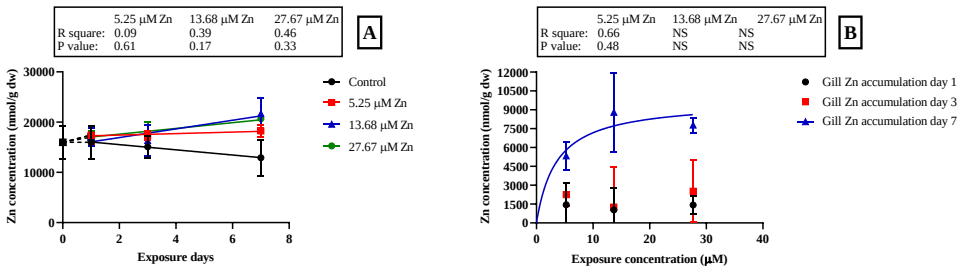
Acknowledgments

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

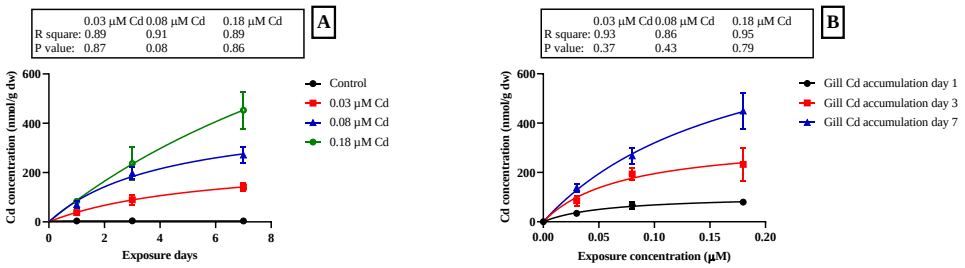
2.6. Supplementary information



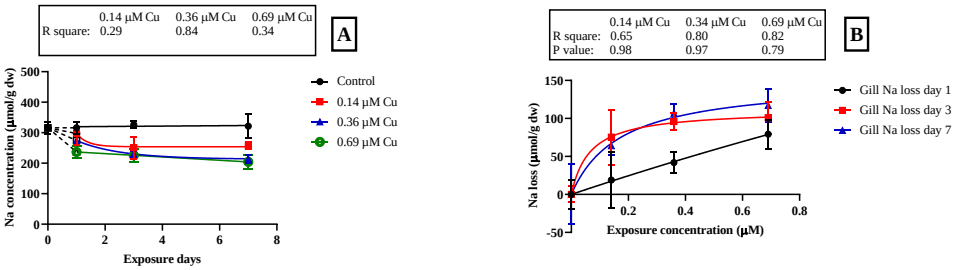
SI-Fig 1: (A) Cu concentration over time, dotted lines indicate assumed accumulation rates during the first day, extrapolating starting values from control values at day 1; (B) net Cu concentration as function of the concentration.



SI-Fig 2: (A) Zn concentration over time, dotted lines indicate assumed accumulation rates during the first day, extrapolating starting values from control values at day 1; (B) net Zn concentration as function of the concentration.



SI-Fig 3: (A) Cd concentration over time, dotted lines indicate assumed accumulation rates during the first day, extrapolating starting values from control values at day 1; (B) net Cd concentration as function of the concentration.



SI-Fig 4: (A) Na concentration over time, dotted lines indicate assumed accumulation rates during the first day, extrapolating starting values from control values at day 1; (B) net Na loss as function of the concentration.

SI-table 1: Total metal concentrations, measured with 7700x ICP-MS (Agilent Technologies, Santa Clara, CA, USA) in the exposure water, expressed in µM (mean ± SD, N=70), BMQL = below minimum quantification limit.

	Cu	**Zn**	**Cd**
Control	0.01 ± 0.002	BMQL	BMQL
25% 96 h LC_{50}	0.14 ± 0.02	5.25 ± 1.06	0.03 ± 0.01
50% 96 h LC_{50}	0.36 ± 0.05	13.68 ± 2.70	0.08 ± 0.03
100% 96 h LC_{50}	0.69 ± 0.12	27.67 ± 4.43	0.18 ± 0.07

SI-table 2: Chemical speciation in the exposure media, calculated using measured concentrations with the equilibrium speciation code VMinteq..

Component	**% of total concentration**	**Species name**
Cu^{+2}	5.862	Cu^{+2}
	9.405	$CuOH^{+}$
	0.868	$Cu(OH)_2$ (aq)
	0.565	$CuSO_4$ (aq)
	82.112	$CuCO_3$ (aq)
	0.254	$CuHCO_3^{+}$
	0.919	$Cu(CO3)_2^{-2}$
Zn^{+2}	73.636	Zn^{+2}
	0.015	$Zn(CO3)_2^{-2}$
	3.255	$ZnOH^{+}$
	4.483	$Zn(OH)_2$ (aq)
	6.885	$ZnSO_4$ (aq)
	0.049	$Zn(SO4)_2^{-2}$
	10.072	$ZnCO_3$ (aq)
	1.595	$ZnHCO_3^{+}$
Cd^{+2}	84.377	Cd^{+2}
	0.298	$CdOH^{+}$
	0.360	$CdCl^{+}$
	8.317	$CdSO_4$ (aq)
	0.093	$Cd(SO4)_2^{-2}$
	1.833	$CdHCO_3^{+}$
	4.705	$CdCO_3$ (aq)
	0.014	$Cd(CO_3)_2^{-2}$

SI-table 3: Primer sequences (F= forward; R= reverse) and Tm°C of target and housekeeping genes.

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	
H^+-ATPase	Sinha et al. (2016)	F - CTATGGGGGTCAACATGGAG	62	103
		R - CCAACACGTGCTTCTCACAC	62	
MT	Reynders et al. (2006b)	F - CCAAGACTGGAACTTGC	52	93
		R - ACGTTGACCTCCTCAC	50	
CAT	Wu et al. (2014)	F - CTGGAAGTGGAATCCGTTTG	60	92
		R - CGACCTCAGCGAAATAGTTG	60	
GST	Casatta et al. (2017)	F - GAAACCAGTTGAGCCGTGC	56	97
		R - CGGCTGGAGAAACTTGCTGAT	57	
GR	Wu et al. (2014)	F - GAGAAGTACGACACCATCCA	53	106
		R - CACACCTATTGAACTGAGATTGAG	54	
CuZnSOD	Wu et al. (2014)	F - TGGCGAAGAAGGCTGTTTGT	60	93
		R - TTCACTGGAGACCCGTCACT	62	

SI-table 4: Cu exposure, metals content (nmol/g dw) and electrolytes level in gills (µmol/g dw) in *Cyprinus carpio* at day 1, 3 and 7. Mean ± SD, N=5, letters indicate significant differences ($p < 0.05$). 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 ($p < 0.05$).

		Cu exposure											
		Day 1				Day 3				Day 7			
		Control	T1	T2	T3	Control	T1	T2	T3	Control	T1	T2	T3
Gill	Cu	70 ± 4 ^{Aa}	187 ± 21 ^{Ba}	194 ± 41 ^{Ba}	235 ± 54 ^{Ba}	75 ± 9 ^{Aa}	205 ± 36 ^{Ba}	270 ± 35 ^{Ba}	270 ± 42 ^{Ba}	85 ± 25 ^{Aa}	267 ± 17 ^{Ba}	393 ± 64 ^{Cb}	512 ± 81 ^{Db}
	Zn	13448 ± 1381	13876 ± 1432	15667 ± 3154	14140 ± 1818	13599 ± 1458	13771 ± 1776	14260 ± 1231	13471 ± 868	14600 ± 841	14576 ± 516	14552 ± 2507	13702 ± 782
	Na	316 ± 18 ^{Aa}	297 ± 3 ^{Aa}	274 ± 13 ^{ABa}	237 ± 19 ^{Ba}	326 ± 10 ^{Aa}	250 ± 36 ^{Ba}	230 ± 10 ^{Bab}	225 ± 21 ^{Ba}	321 ± 39 ^{Aa}	257 ± 11 ^{Ba}	214 ± 12 ^{BCb}	204 ± 22 ^{Ca}
	K	291 ± 13	298 ± 13	287 ± 12	286 ± 19	293 ± 7	276 ± 19	285 ± 8	284 ± 16	263 ± 54	284 ± 9	272 ± 24	286 ± 11
	Ca	561 ± 199	559 ± 57	583 ± 54	599 ± 142	617 ± 114	578 ± 66	617 ± 72	557 ± 70	648 ± 167	579 ± 41	639 ± 102	552 ± 47
	Mg	52 ± 1 ^{Aa}	52 ± 1 ^{Aa}	53 ± 3 ^{Aa}	56 ± 3 ^{Aa}	58 ± 4 ^{ABa}	51 ± 5 ^{Aa}	62 ± 3 ^{BCb}	66 ± 2 ^{Cb}	55 ± 2 ^{Aa}	54 ± 1 ^{Aa}	59 ± 5 ^{Aab}	60 ± 3 ^{Aab}

SI-table 5*:* Zn exposure, metals content (nmol/g dw) and electrolytes level in gills (µmol/g dw) in *Cyprinus carpio* at day 1, 3 and 7. Mean ± SD, N=5, letters indicate significant differences ($p < 0.05$). 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 ($p < 0.05$).

		Zn exposure											
		Day 1				Day 3				Day 7			
		Control	T1	T2	T3	Control	T1	T2	T3	Control	T1	T2	T3
Gill	Cu	83 ± 28	89 ± 48	79 ± 16	65 ± 6	67 ± 4	75 ± 5	74 ± 19	64 ± 10	62 ± 7	81 ± 12	75 ± 4	8 ∓ 59
	Zn	15990 ± 3315 ^{Aa}	17433 ± 1725 ^{Aa}	17024 ± 1767 ^{Ab}	17410 ± 718 ^{Aa}	15081 ± 2237 ^{Aa}	17324 ± 929 ^{Aa}	17495 ± 2136 ^{Aa}	17608 ± 2476 ^{Aa}	12911 ± 3594 ^{Aa}	18246 ± 1130 ^{Ba}	21716 ± 3134 ^{Bb}	20694 ± 598 ^{Ba}
	Na	326 ± 48	339 ± 31	316 ± 20	295 ± 34	330 ± 33	334 ± 18	318 ± 75	309 ± 47	256 ± 103	339 ± 28	341 ± 25	328 ± 43
	K	295 ± 49	300 ± 15	292 ± 16	268 ± 31	289 ± 16	307 ± 13	290 ± 74	286 ± 41	210 ± 105	302 ± 16	298 ± 16	294 ± 61
	Ca	651 ± 114	641 ± 132	616 ± 46	590 ± 41	658 ± 76	682 ± 52	609 ± 147	579 ± 70	656 ± 207	685 ± 30	719 ± 67	645 ± 159
	Mg	56 ± 9	54 ± 5	55 ± 4	52 ± 3	51 ± 3	55 ± 3	50 ± 12	52 ± 7	43 ± 13	52 ± 1	56 ± 4	54 ± 8

SI-table 6*:* Cd exposure, metals content (nmol/g dw) and electrolytes level in gills (µmol/g dw) in *Cyprinus carpio* at day 1, 3 and 7. Mean ± SD, N=5, letters indicate significant differences ($p < 0.05$). 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 ($p < 0.05$).

		Cd exposure											
		Day 1				Day 3				Day 7			
		Control	T1	T2	T3	Control	T1	T2	T3	Control	T1	T2	T3
Gill	Cd	3.7 ± 1 ^{Aa}	37.7 ± 8.1 ^{Ba}	70.4 ± 13.2 ^{Ca}	83.2 ± 5.7 ^{Ca}	4.2 ± 1.2 ^{Aa}	89.7 ± 21.2 ^{Bb}	197.2 ± 24.5 ^{Cb}	236.8 ± 67.4 ^{Cb}	3.8 ± 0.5 ^{Aa}	141.4 ± 16.8 ^{Bc}	271.2 ± 31.9 ^{Cb}	452.8 ± 74.6 ^{Dc}
	Cu	67 ± 14	73 ± 6	70 ± 6	74 ± 6	80 ± 8	75 ± 14	86 ± 4	73 ± 12	83 ± 5	85 ± 19	88 ± 9	83 ± 4
	Zn	16974 ± 4432	18918 ± 2439	19098 ± 3796	17395 ± 615	19790 ± 3540	19235 ± 4503	16542 ± 1570	17368 ± 3839	18928 ± 987	19811 ± 3371	18439 ± 1506	17728 ± 3145
	Na	412 ± 93	459 ± 26	444 ± 32	410 ± 13	448 ± 22	410 ± 101	456 ± 32	432 ± 51	461 ± 29	477 ± 81	471 ± 45	470 ± 27
	K	341 ± 56	378 ± 7	369 ± 15	358 ± 14	374 ± 16	345 ± 69	383 ± 11	366 ± 32	369 ± 39	374 ± 33	376 ± 21	403± 13
	Ca	845 ± 95	935 ± 144	835 ± 154	738 ± 75	899 ± 99	793 ± 187	747 ± 93	740 ± 170	868 ± 101	853 ± 131	828 ± 206	829 ± 106
	Mg	56 ± 7	67 ± 6	60 ± 7	54 ± 3	65 ± 3	60 ± 8	62 ± 2	54 ± 4	58 ± 3	60 ± 9	59± 7	59 ± 3

SI-table 7: total metal accumulation, % increase relative to control and accumulation rate in Cyprinus carpio *gills exposed to different Cu, Zn and Cd concentrations for 1, 3 and 7 days (mean ± SD).*

Cu exposure												
	Day 1				Day 3				Day 7			
Exposure	Control	0.14 µM	0.36 µM	0.69 µM	Control	0.14 µM	0.36 µM	0.69 µM	Control	0.14 µM	0.36 µM	0.69 µM
Total metal content (nmol/g dw)	70 ± 4	187 ± 21	194 ± 41	235 ± 54	74 ± 9	205 ± 36	270 ± 35	270 ± 42	85 ± 25	267 ± 17	393 ± 64	512 ± 81
Net accumulation (nmol/g dw)	/	117 ± 20	124 ± 40	165 ± 54	/	130 ± 36	195 ± 35	195 ± 42	/	182 ± 17	308 ± 63	427 ± 81
% increase	/	168 ± 29	178 ± 58	236 ± 77	/	174 ± 48	261 ± 47	261 ± 56	/	213 ± 20	362 ± 74	501 ± 95
Accumulation rate (nmol/g dw/h)	/	4.9 ± 0.9	5.2 ± 1.7	6.9 ± 2.3	/	1.8 ± 0.5	2.7 ± 0.5	2.7 ± 0.6	/	1.1 ± 0.1	1.8 ± 0.4	2.5 ± 0.5
Zn exposure												
	Day 1				Day 3				Day 7			
Exposure concentration	Control	5.25 µM	13.68 µM	27.67 µM	Control	5.25 µM	13.68 µM	27.67 µM	Control	5.25 µM	13.68 µM	27.67 µM
Total metal content (nmol/g dw)	15990 ± 3315	17433 ± 1725	17024 ± 1767	17410 ± 718	15081 ± 2237	17324 ± 929	17495 ± 2136	17608 ± 2476	12911 ± 3594	18246 ± 1130	21716 ± 3134	20694 ± 598
Net accumulation (nmol/g dw)	/	1443 ± 1725	1034 ± 1767	1420 ± 718	/	2243 ± 928	2414 ± 3161	2528 ± 2476	/	5335 ± 1130	8805 ± 3134	7783 ± 597
% increase	/	9 ± 10	6 ± 11	8 ± 4	/	15 ± 6	8 ± 20	16 ± 16	/	41 ± 8	68 ± 24	60 ± 4
Accumulation rate (nmol/g dw/h)	/	60 ± 71	43 ± 73	59 ± 29	/	31 ± 12	17 ± 43	35 ± 34	/	31 ± 6	52 ± 18	46 ± 3
Cd exposure												
	Day 1				Day 3				Day 7			
Exposure concentration	Control	0.03 µM	0.08 µM	0.18 µM	Control	0.03 µM	0.08 µM	0.18 µM	Control	0.03 µM	0.08 µM	0.18 µM
Total metal content (nmol/g dw)	3.6 ± 1	37 ± 8	70 ± 13	83 ± 5	4 ± 1	89 ± 21	197 ± 24	236 ± 67	3.7 ± 0.5	141 ± 16	271 ± 31	452 ± 74
Net accumulation (nmol/g dw)	/	34 ± 8	66 ± 13	79 ± 5	/	85 ± 21	193 ± 24	232 ± 67	/	137 ± 16	267 ± 31	449 ± 74
% increase	/	923 ± 221	1793 ± 358	2146 ± 154	/	2044 ± 506	4618 ± 586	5550 ± 1611	/	3658 ± 445	7109 ± 847	11936 ± 1981
Accumulation rate (nmol/g dw/h)	/	1.41 ± 0.3	2.7 ± 0.5	3.3 ± 0.2	/	1.2 ± 0.3	2.7 ± 0.3	3.2 ± 0.9	/	0.8 ± 0.1	1.6 ± 0.2	2.7 ± 0.4

SI-table 8: relative mRNA abundance in Cyprinus carpio gills and 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 ($p < 0.05$).

		Cu exposure											
		Day 1				Day 3				Day 7			
		Control	T1	T2	T3	Control	T1	T2	T3	Control	T1	T2	T3
Gill	CAT	1.03 ± 0.28 ^{Aa}	0.46 ± 0.12 ^{A}	1.06 ± 0.14 ^{AB}	0.7 ± 0.38 ^{B}	1.00 ± 0.1 ^{A}	0.67 ± 0.14 ^{A}	0.93 ± 0.03 ^{A}	1.01 ± 0.08 ^{A}	1.04 ± 0.30 ^{A}	0.9 ± 0.11 ^{A}	1.11 ± 0.07 ^{A}	1.13 ± 0.14 ^{A}
	CuZnSOD	1.05 ± 0.39 ^{Aa}	0.8 ± 0.09 ^{ABa}	0.69 ± 0.12 ^{ABa}	0.62 ± 0.05 ^{Ba}	1.00 ± 0.08 ^{Aa}	1.19 ± 0.14 ^{Aab}	1.34 ± 0.15 ^{Ab}	1.17 ± 0.05 ^{Ab}	1.00 ± 0.13 ^{Aa}	1.23 ± 0.11 ^{Ab}	1.36 ± 0.14 ^{Ab}	1.23 ± 0.14 ^{Ab}
	GR	1.03 ± 0.28 ^{A}	1.49 ± 0.29 ^{AB}	2.02 ± 0.61 ^{B}	1.81 ± 0.12 ^{B}	1.00 ± 0.20 ^{A}	1.07 ± 0.11 ^{A}	1.82 ± 0.18 ^{AB}	1.87 ± 0.42 ^{B}	1.00 ± 0.14 ^{A}	1.25 ± 0.20 ^{AB}	1.87 ± 0.28 ^{B}	2.445 ± 0.33 ^{B}
	GST	1.01 ± 0.17 ^{A}	1.16 ± 0.24 ^{A}	0.61 ± 0.30 ^{BC}	0.46 ± 0.15 ^{C}	1.00 ± 0.12 ^{AB}	1.18 ± 0.15 ^{A}	1.08 ± 0.25 ^{AB}	0.68 ± 0.15 ^{B}	1.01 ± 0.16 ^{A}	1.16 ± 0.13 ^{A}	0.98 ± 0.22 ^{A}	0.82 ± 0.16 ^{A}
	MT	1.13 ± 0.71 ^{Aa}	2.65 ± 0.50 ^{Ba}	2.82 ± 0.65 ^{Ba}	4.45 ± 0.60 ^{Ca}	1.01 ± 0.20 ^{Aa}	1.524 ± 0.29 ^{ABa}	2.70 ± 0.63 ^{BCa}	2.8 ± 0.39 ^{Cb}	1.01 ± 0.17 ^{Aa}	1.53 ± 0.21 ^{ABa}	1.87 ± 0.38 ^{ABa}	2.42 ± 0.62 ^{Bb}
	H^+-ATPase	1.00 ± 0.07 ^{Aa}	1.46 ± 0.06 ^{Ba}	1.90 ± 0.16 ^{Cb}	2.29 ± 0.06 ^{Db}	1.00 ± 0.11 ^{Aa}	1.13 ± 0.10 ^{ABb}	1.23 ± 0.08 ^{ABb}	1.28 ± 0.08 ^{Bb}	1.00 ± 0.12 ^{Aa}	0.99 ± 0.11 ^{Ab}	1.10 ± 0.14 ^{Ab}	1.20 ± 0.07 ^{Ab}
	Na^+/K^+-ATPase	1.00 ± 0.07 ^{Aa}	1.02 ± 0.12 ^{Aa}	1.25 ± 0.24 ^{ABa}	1.33 ± 0.09 ^{Ba}	1.01 ± 0.15 ^{Aa}	0.69 ± 0.02 ^{Bb}	0.57 ± 0.10 ^{Bb}	0.70 ± 0.10 ^{Bb}	1.00 ± 0.10 ^{Aa}	0.76 ± 0.09 ^{Aab}	0.80 ± 0.04 ^{Ab}	0.89 ± 0.10 ^{Ab}

		Cu exposure											
		Day 1				Day 3				Day 7			
		Control	T1	T2	T3	Control	T1	T2	T3	Control	T1	T2	T3
Liver	CAT	1.02 ± 0.28 ^{Aa}	1.00 ± 0.6 ^{Aa}	0.70 ± 0.03 ^{ABa}	0.33 ± 0.02 ^{Ba}	1.04 ± 0.35 ^{Aa}	0.85 ± 0.26 ^{Aa}	0.75 ± 0.19 ^{Aa}	0.56 ± 0.12 ^{Aab}	1.24 ± 0.19 ^{Aa}	0.91 ± 0.11 ^{Aa}	0.97 ± 0.05 ^{Aa}	0.98 ± 0.17 ^{Aa}
	CuZnSOD	1.01 ± 0.20	0.88 ± 0.10	0.91 ± 0.05	0.84 ± 0.24	1.01 ± 0.19	1.04 ± 0.23	1.07 ± 0.07	0.95 ± 0.14	1.00 ± 0.11	0.80 ± 0.07	0.97 ± 0.11	0.95 ± 0.19
	GR	1.03 ± 0.31	0.68 ± 0.02	0.75 ± 0.26	0.98 ± 0.23	1.04 ± 0.22	0.83 ± 0.23	0.70 ± 0.17	0.72 ± 0.35	1.10 ± 0.48	1.14 ± 0.22	1.28 ± 0.30	1.31 ± 0.22
	GST	1.08 ± 0.47 ^{Aa}	0.88 ± 0.16 ^{Aa}	0.66 ± 0.08 ^{ABb}	0.40 ± 0.11 ^{Ba}	1.02 ± 0.23 ^{Aa}	0.50 ± 0.14 ^{Ba}	0.46 ± 0.15 ^{Bab}	0.20 ± 0.04 ^{Ba}	1.02 ± 0.28 ^{ABa}	1.17 ± 0.14 ^{Aa}	1.22 ± 0.16 ^{Ab}	0.63 ± 0.07 ^{Ba}
	MT	1.00 ± 0.08 ^{ABa}	1.62 ± 0.06 ^{Aab}	1.31 ± 0.31 ^{ABa}	0.78 ± 0.10 ^{Ba}	1.02 ± 0.27 ^{Aa}	2.12 ± 0.42 ^{Ba}	1.85 ± 0.33 ^{Ba}	3.02 ± 0.64 ^{Cb}	1.03 ± 0.30 ^{Aa}	1.28 ± 0.18 ^{Ab}	1.37 ± 0.43 ^{Aa}	1.64 ± 0.22 ^{Ac}

SI-table 9: relative mRNA abundance in *Cyprinus carpio* gills and liver exposed to different Cd 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 ($p < 0.05$).

		Cd exposure											
		Day 1				Day 3				Day 7			
		Control	T1	T2	T3	Control	T1	T2	T3	Control	T1	T2	T3
Gill	CAT	1.01 ± 0.12	0.99 ± 0.05	1.02 ± 0.15	0.97 ± 0.05	1.00 ± 0.09	1.02 ± 0.07	1.07 ± 0.08	1.19 ± 0.12	1.00 ± 0.09	0.82 ± 0.10	0.86 ± 0.09	0.88 ± 0.08
	CuZnSOD	1.01 ± 0.12	1.02 ± 0.16	1.04 ± 0.15	1.01 ± 0.06	1.00 ± 0.05	1.02 ± 0.08	1.05 ± 0.10	1.12 ± 0.17	1.02 ± 0.20	1.00 ± 0.04	1.08 ± 0.15	0.92 ± 0.08
	GR	1.02 ± 0.21^{ABa}	0.97 ± 0.14^{Ba}	1.38 ± 0.10^{Aa}	1.20 ± 0.15^{ABab}	1.02 ± 0.23^{Aa}	1.04 ± 0.15^{Aa}	1.13 ± 0.08^{Aa}	1.54 ± 0.13^{Bb}	1.00 ± 0.11^{Aa}	1.04 ± 0.22^{Aa}	1.22 ± 0.06^{Aa}	1.13 ± 0.15^{Aa}
	GST	1.00 ± 0.11	0.90 ± 0.15	1.03 ± 0.26	0.97 ± 0.19	1.00 ± 0.10	1.00 ± 0.08	0.97 ± 0.06	0.94 ± 0.14	1.01 ± 0.15	0.87 ± 0.03	0.89 ± 0.12	0.84 ± 0.13
	MT	1.01 ± 0.13^{A}	3.44 ± 0.86^{B}	4.59 ± 1.99^{B}	4.85 ± 0.72^{B}	1.02 ± 0.23^{A}	4.08 ± 0.26^{B}	4.97 ± 0.87^{B}	4.25 ± 0.44^{B}	1.01 ± 0.15^{A}	4.39 ± 0.87^{B}	5.08 ± 0.43^{B}	5.38 ± 1.39^{B}
	H^+-ATPase	1.02 ± 0.24	0.92 ± 0.11	1.01 ± 0.17	0.92 ± 0.08	1.03 ± 0.28	1.12 ± 0.20	1.22 ± 0.17	1.23 ± 0.27	1.01 ± 0.19	1.18 ± 0.25	1.27 ± 0.11	1.41 ± 0.29
	Na^+/K^+-ATPase	1.01 ± 0.16	0.78 ± 0.09	0.89 ± 0.17	0.76 ± 0.09	1.01 ± 0.15	1.12 ± 0.17	1.25 ± 0.21	1.21 ± 0.23	1.01 ± 0.19	0.85 ± 0.15	0.81 ± 0.04	0.75 ± 0.07

		Cd exposure											
		Day 1				Day 3				Day 7			
		Control	T1	T2	T3	Control	T1	T2	T3	Control	T1	T2	T3
Liver	CAT	1.02 ± 0.24	1.48 ± 0.53	0.99 ± 0.16	1.18 ± 0.14	1.00 ± 0.10	1.18 ± 0.09	0.99 ± 0.11	1.09 ± 0.32	1.01 ± 0.12	1.08 ± 0.20	0.94 ± 0.14	1.09 ± 0.13
	CuZnSOD	1.02 ± 0.21	1.52 ± 0.65	1.12 ± 0.21	1.23 ± 0.14	1.01 ± 0.15	0.87 ± 0.09	0.93 ± 0.28	0.94 ± 0.21	1.01 ± 0.12	1.05 ± 0.28	0.84 ± 0.16	1.22 ± 0.44
	GR	1.01 ± 0.16	1.81 ± 0.84	1.16 ± 0.61	1.32 ± 0.11	1.02 ± 0.22	1.08 ± 0.34	0.87 ± 0.42	1.03 ± 0.17	1.01 ± 0.17	1.03 ± 0.21	0.96 ± 0.11	1.02 ± 0.43
	GST	0.93 ± 0.32	0.98 ± 0.32	0.88 ± 0.32	1.15 ± 0.30	1.04 ± 0.32	1.26 ± 0.20	1.26 ± 0.25	1.14 ± 0.44	1.02 ± 0.23	1.11 ± 0.34	1.06 ± 0.16	1.22 ± 0.33
	MT	1.00 ± 0.09^{Aa}	1.00 ± 0.42^{Aa}	1.95 ± 0.61^{Ba}	2.52 ± 0.54^{Ba}	1.04 ± 0.19^{Aa}	1.12 ± 0.27^{Aa}	0.88 ± 0.27^{Ab}	1.35 ± 0.04^{Ab}	1.04 ± 0.33^{Aa}	1.24 ± 0.30^{Aa}	0.75 ± 0.38^{Ab}	1.15 ± 0.10^{Ab}

SI-table 10: relative mRNA abundance in *Cyprinus carpio* gills and liver exposed to different Zn 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 ($p < 0.05$).

		Zn exposure											
		Day 1				Day 3				Day 7			
		Control	T1	T2	T3	Control	T1	T2	T3	Control	T1	T2	T3
Gill	CAT	1.01 ± 0.16	0.97 ± 0.15	1.02 ± 0.02	0.94 ± 0.05	1.00 ± 0.10	1.08 ± 0.14	1.13 ± 0.12	1.05 ± 0.08	1.01 ± 0.13	0.82 ± 0.17	0.92 ± 0.13	0.86 ± 0.13
	CuZnSOD	1.01 ± 0.12	1.02 ± 0.14	1.18 ± 0.14	1.02 ± 0.15	1.01 ± 0.13	1.12 ± 0.15	0.98 ± 0.10	1.00 ± 0.14	1.01 ± 0.18	0.84 ± 0.21	1.08 ± 0.13	0.97 ± 0.16
	GR	1.00 ± 0.09	0.80 ± 0.07	1.14 ± 0.08	0.90 ± 0.11	1.00 ± 0.09	1.05 ± 0.10	1.09 ± 0.16	1.17 ± 0.19	1.02 ± 0.20	1.03 ± 0.27	1.01 ± 0.12	0.93 ± 0.07
	GST	1.03 ± 0.31	0.86 ± 0.17	0.96 ± 0.16	0.84 ± 0.18	1.00 ± 0.09	1.42 ± 0.23	1.05 ± 0.16	1.11 ± 0.11	1.01 ± 0.17	1.01 ± 0.13	0.96 ± 0.09	0.91 ± 0.14
	MT	1.04 ± 0.34 ^{Aa}	3.24 ± 0.17 ^{Ba}	4.99 ± 1.34 ^{Ca}	3.34 ± 0.30 ^{Ba}	1.00 ± 0.07 ^{Aa}	3.00 ± 0.53 ^{Ba}	4.12 ± 0.54 ^{Bab}	5.99 ± 0.87 ^{Cb}	1.02 ± 0.20 ^{Aa}	2.00 ± 0.34 ^{Aa}	3.38 ± 0.21 ^{BCb}	4.84 ± 1.22 ^{Cab}
	H^+-ATPase	1.01 ± 0.14	0.92 ± 0.14	0.95 ± 0.07	0.85 ± 0.12	1.00 ± 0.09	1.00 ± 0.09	0.99 ± 0.14	0.95 ± 0.13	1.00 ± 0.08	0.87 ± 0.19	0.91 ± 0.16	0.88 ± 0.18
	Na^+/K^+-ATPase	1.01 ± 0.19	1.00 ± 0.24	0.96 ± 0.08	0.72 ± 0.14	1.00 ± 0.06	0.98 ± 0.20	0.98 ± 0.13	1.01 ± 0.10	1.01 ± 0.11	1.02 ± 0.18	1.06 ± 0.19	1.15 ± 0.15

		Zn exposure											
		Day 1				Day 3				Day 7			
		Control	T1	T2	T3	Control	T1	T2	T3	Control	T1	T2	T3
Liver	CAT	1.00 ± 0.10	1.16 ± 0.27	1.02 ± 0.19	1.03 ± 0.19	1.02 ± 0.19	1.02 ± 0.13	1.13 ± 0.07	1.17 ± 0.15	1.01 ± 0.18	0.91 ± 0.01	0.90 ± 0.2	0.88 ± 0.16
	CuZnSOD	1.01 ± 0.12	1.00 ± 0.31	0.94 ± 0.17	0.96 ± 0.21	1.00 ± 0.07	1.08 ± 0.10	1.01 ± 0.04	1.22 ± 0.12	1.00 ± 0.01	0.90 ± 0.07	0.99 ± 0.10	0.89 ± 0.05
	GR	1.03 ± 0.26	0.83 ± 0.28	0.87 ± 0.18	0.67 ± 0.09	1.01 ± 0.16	1.00 ± 0.25	1.24 ± 0.21	1.22 ± 0.28	1.08 ± 0.51	0.71 ± 0.15	0.82 ± 0.43	0.70 ± 0.05
	GST	1.02 ± 0.20	1.12 ± 0.14	1.23 ± 0.18	1.66 ± 0.72	1.00 ± 0.08	1.13 ± 0.43	1.18 ± 0.24	1.05 ± 0.19	1.01 ± 0.15	0.85 ± 0.16	0.82 ± 0.15	0.96 ± 0.42
	MT	1.11 ± 0.55 ^{A}	1.42 ± 0.94 ^{A}	2.61± 1.84 ^{A}	2.7 ± 1.81 ^{A}	0.86 ± 0.08 ^{A}	0.97 ± 0.47 ^{AB}	1.50 ± 0.37 ^{AB}	3.61 ± 0.32 ^{B}	1.00 ± 0.11 ^{A}	1.75 ± 0.85 ^{AB}	2.00 ± 1.28 ^{AB}	3.71 ± 1.24 ^{B}

SI-table 11: Gill tissue Na net loss, % of loss and loss rate for *Cyprinus carpio* exposed to different concentrations of Cu for 1, 3 and 7 days.

Na loss												
	Day 1				Day3				Day 7			
Cu exposure concentration	Control	0.14 µM	0.36 µM	0.69 µM	Control	0.14 µM	0.36 µM	0.69 µM	Control	0.14 µM	0.36 µM	0.69 µM
Total Na concentration (µmol/g dw)	316 ± 18	297 ± 37	274 ± 13	237 ± 19	326 ± 10	250 ± 36	230 ± 10	225 ± 21	321 ± 39	257 ± 11	214 ± 12	204 ± 22
Net Na loss (µmol/g dw)	/	19 ± 37	42 ± 13	79 ± 19	/	76 ± 36	96 ± 10	101 ± 21	/	64 ± 11	107 ± 12	117 ± 22
% Na loss	/	6 ± 11	13 ± 4	25 ± 6	/	23 ± 11	29 ± 3	31 ± 6	/	20 ± 3	33 ± 3	36 ± 6
Rate of Na loss (µmol/g dw/h)	/	0.79 ± 1.55	1.75 ± 0.56	3.30 ± 0.79	/	1.05 ± 0.50	1.33 ± 0.14	1.40 ± 0.30	/	0.38 ± 0.07	0.63 ± 0.07	0.70 ± 0.13

Chapter 3.

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

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

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

Modified version of Aquatic Toxicology (2020); 226, 105561

Abstract

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

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

3.1. Introduction

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

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

The antioxidant defence system plays a crucial role in preventing deleterious effects caused by reactive oxygen species (ROS). Enzymes such as SOD, and catalase (CAT) represent the first line of defence, converting superoxide ($O_2^{-\bullet}$) into hydrogen peroxide (H_2O_2), and H_2O_2 into water (H_2O) and oxygen (O_2) (Pillet et al. 2019). Furthermore, GSH plays an important role in ROS defence and as a chelating agent for metal ions (Lange et al. 2002). Thus, the presence of enzymes such glutathione reductase (GR) and glutathione-S-transferase (GST) is needed for glutathione metabolism. Glutathione-S-transferase metabolizes lipid hydroperoxides (Dautremepuits et al. 2009) and mediates the conjugation reaction of GSH with electrophilic compounds, causing the depletion of GSH (Dickinson and Forman 2002). Glutathione reductase catalyses the reduction of glutathione disulfide (GSSG) in order to maintain a constant ratio of

GSH/GSSG (Couto et al. 2016). In addition to the antioxidant system, metallothioneins (MT) play an important role in protecting the organism from metal toxicity. Metallothioneins are cysteine rich proteins, that play a significant role in essential metal ion homeostasis (e.g. Zn and Cu) and binding of non-essential metal ions (e.g. Cd) for sequestration (Atli and Canli 2008, Jakimska et al. 2011). Their levels and activity in tissues can be stimulated by both essential and non-essential metal ions (Hogstrand and Haux 1990, Wu et al. 1999).

Organisms in aquatic ecosystems are generally exposed to a mixture of metals that can be taken up via common uptake routes and interact with each other during uptake (Komjarova and Blust 2009). This interaction can stimulate or inhibit the uptake of particular compounds. For example, a non-competitive interaction can occur between Cu and silver (Ag) in which Ag can stimulate gill-Cu binding but not the other way around(Niyogi et al. 2015). A competitive interaction on the other hand can occur between Cd^{2+} and Zn^{2+}, since they have a comparable electron configuration and they both have a high affinity for thiol groups (Brzóska and Moniuszko-Jakoniuk 2001). Cadmium uptake can also be inhibited in presence of Cu as demonstrated in several organisms, such as zebrafish, rainbow trout and freshwater mussel (*Pyganodon grandis*) (Stewart 1999, Franklin et al. 2002, Kamunde and MacPhail 2011, Komjarova and Bury 2014). Deleterious effects of mixtures of these metal ions have already been studied in different model species such as Mediterranean mussel (*Mytilus galloprovincialis*) (Benali et al. 2017), rainbow trout (*Oncorhynchus mykiss*) (Kamunde and MacPhail 2011a) and zebrafish (*Danio rerio*) (Komjarova and Bury 2014).

The aim of the present study was to investigate bioaccumulation, ionoregulation and responses of defensive mechanisms in common carp after a short-term exposure to waterborne binary mixtures of Cu(II) and Cd(II), using environmentally relevant concentrations. In addition, possible interactions between the two metal ions were investigated. Therefore, common carp were exposed to a series of sublethal mixtures of Cu (nominal concentrations used: 0.07, 0.19 and 0.38 µM) and Cd (nominal concentrations used: 0.02, 0.05, 0.10 µM) using environmentally relevant concentrations. In Flanders (Belgium) where this study was conducted, the water quality guideline for dissolved Cu in surface water is set to 0.11 µM, whereas for Cd the maximum values ranged between 0.004 to 0.013 µM depending on water hardness (Belgian Official Journal, 2015). However, in reality these limits are often exceeded. For instance, in Flanders the Flemish Environmental Agency (VMM) has measured concentration up to 2.05 µM for Cu and 1.06 µM for Cd (VMM 2016). Furthermore, a field study done in Flanders over 14 different locations reported dissolved metal concentrations up to 0.4 µM and 0.2 µM for Cu and Cd, respectively (Bervoets and Blust 2003). Therefore, the metal concentrations used in this study can be considered as environmentally relevant.

With this work, together with all the previous studies investigating and showing the complexity of metal mixture scenarios (Komjarova and Blust 2009, Niyogi et al. 2015, Brix et al. 2017, Pillet et al. 2019) we aim to provide new insights into understanding

metal accumulation and toxicity in multi-metal exposure scenarios. We hypothesize that metal mixtures would remain sub-lethal, as our exposures were relatively short and maximum exposure concentrations were 25 % + 50 % of the 96h-LC_{50}. Furthermore, we anticipate a quick metal bioaccumulation for both Cu and Cd, even though a reduced accumulation of Cd is expected in presence of Cu. Moreover, we expect that metal accumulation would trigger defensive mechanisms, such as MT and GR to mitigate possible deleterious effects. Regarding ion-homeostasis, in agreement with previous results from our lab, we expect a Na but not a Ca loss (Castaldo et al. 2020c, Delahaut et al. 2020).

3.2. Material and methods

3.2.1. Experimental animals

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

3.2.2. Experimental set-up

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

nominal and measured metal concentrations in the water for both exposure series are shown in Table 1 and 2. Metal speciation was calculated with VMinteq (Supplementary information, SI-Table 1 and SI-Table 2).

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

	Nominal concentration	**Measured concentration**
Control	0 µM Cu 0 µM Cd	0.0031 ± 0.0031 µM Cu < 0.00089 µM (BMQL) Cd
Treatment Cu_{fix}/Cd_{10}	25 % LC_{50} Cu (0.19 µM) 10 % LC_{50} Cd (0.02 µM)	0.15 ± 0.020 µM Cu 0.025 ± 0.0027 µM Cd
Treatment Cu_{fix}/Cd_{25}	25 % LC_{50} Cu (0.19 µM) 25 % LC_{50} Cd (0.05 µM)	0.16 ± 0.020 µM Cu 0.062 ± 0.0062 µM Cd
Treatment Cu_{fix}/Cd_{50}	25 % LC_{50} Cu (0.19 µM) 50 % LC_{50} Cd (0.10 µM)	0.16 ± 0.024 µM Cu 0.12 ± 0.012 µM Cd

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

	Nominal concentration	**Measured concentration**
Control	0 µM Cu 0 µM Cd	0.0047 ± 0.0013 µM Cu < 0.00089 µM (BMQL) Cd
Treatment Cd_{fix}/Cu_{10}	10 % LC_{50} Cu (0.077 µM) 25 % LC_{50} Cd (0.05 µM)	0.068 ± 0.011 µM Cu 0.060 ± 0.0062 µM Cd
Treatment Cd_{fix}/Cu_{25}	25 % LC_{50} Cu (0.19 µM) 25 % LC_{50} Cd (0.05 µM)	0.16 ± 0.020 µM Cu 0.062 ± 0.0062 µM Cd
Treatment Cd_{fix}/Cu_{50}	50 % LC_{50} Cu (0.38 µM) 25 % LC_{50} Cd (0.05 µM)	0.34 ± 0.036 µM Cu 0.061 ± 0.0062 µM Cd

3.2.3. Metal bioaccumulation and electrolyte levels

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

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

3.2.4. RNA extraction and real time PCR

The second and the third gill arch of individual fish and an aliquot of the pooled liver samples of two fish were used for RNA extraction and gene expression. Total RNA was extracted from samples (~ 20/30 mg) using Trizol (Invitrogen, Merelbeke, Belgium) following the manufacturer's instructions. Nano-Drop spectrophotometry (NanoDrop Technologies, Wilmington, DE) was used to determine RNA quantity and quality, whereas integrity was evaluated with a 1% agarose gel with ethidium bromide (500 µg/ml). DNase treatment was performed using the commercial RNase free kit DNase I from Thermo Fisher Scientific (Waltham, MA, USA). Then 1 µg of RNA was transcribed to cDNA according to RevertAid H minus First strand cDNA synthesis kit protocol (Thermo fisher, Fermentas, Cambridgeshire). According to the OD260/OD280 nm absorption ratio (higher than 1.8), four samples were selected and used for qPCR. Real-

time PCR was performed using a Mx3005P QPCR System (Agilent Technologies, Belgium). The assay was performed in duplicate in a final reaction volume of 20 µl containing 10 µl of Brilliant III Ultra-Fast QPCR Master Mix (Agilent), 500 nM of each primer (reverse and forward), 5.7 µl of sterile water, 0.3 µl of reference dye and 5 ng of cDNA. PCR amplification was carried out following the Brilliant III Ultra-Fast QPCR Master Mix (Agilent) protocol for Agilent Mx3005P QPCR system. Oligonucleotides primers were taken from literature: elongation factor 1α (eEF) (Sinha et al. 2012), β-actin (Wu et al. 2014); H^+-ATPase (Sinha et al. 2016), catalase (CAT) (Wu et al. 2014) , superoxide dismutase Cu-Zn (SOD) (Wu et al. 2014) , glutathione reductase (GR) (Wu et al. 2014), glutathione S-transferase (GST) (Casatta et al. 2017), metallothionein (MT) (Reynders et al. 2006b), Na^+/H^+-exchanger (NHE-2) (Castaldo et al. 2020c) and Na^+/K^+-ATPase (Castaldo et al. 2020c). Primers for CTR1 were designed using NCBI resources Primer blast and synthesized as highly purified salt-free "OliGold" primers by Eurogentec (Eurogentec, Seraing, Belgium). Primer sequences, annealing temperature and primer efficiency are given in SI-Table 10. Primer efficiency was determined based on the slope of the standard curve, using a serial dilution of cDNA.

3.2.5. Statistical analysis

All data were presented as mean values ± S.D. For the statistical analyses, normality of the data was tested with the Shapiro-Wilk test. Two-way analyses of variance (ANOVA) were performed on the obtained data, followed by Tukey test. Data were considered statistically significant when *p*-value < 0.05. All statistical tests were performed with GraphPad Prism version 8.02 for Windows (GraphPad Software, La Jolla California USA). According to Custer et al. (2000), for metal concentrations below the minimum quantification limit (BMQL), a value of MQL/2 was assigned. If > 50% of the observations were BMQL, no statistical tests were conducted. Data presented in the supplementary information, were analysed using the same software.

3.3. Results

3.3.1. Dynamics of Cu and Cd bioaccumulation

3.3.1.1. Copper bioaccumulation

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

exposure period, a significant increase was observed in the treatments Cu_{fix}/Cd_{10-25} compared with the control. In the treatment Cd_{fix}/Cu_{50}, Cu increased significantly compared with the control from day three onwards. Moreover, at day seven, the metal content was higher in the treatments Cu_{fix}/Cd_{25} and Cd_{fix}/Cu_{25} compared to day one (Fig. 1.1C and 1.2C).

For both experimental series, a fast Cu accumulation was observed in the gills during the first day in all treatments. From day one onwards, Cu accumulation continued at a slightly lower pace, increasing linearly in time (See supplementary information, SI-Fig 1 A and B). During experimental series $Cu_{fix}/Cd_{var,}$ there seems to be a steady Cu net accumulation rate ($\simeq$ 2.6, 1.5 and 1.4 nmol g^{-1} dw h^{-1} for day one, three and seven respectively), which is not affected by Cd levels in the water (SI-Fig 1 A and SI-Table 3). However, looking at experimental series Cd_{fix}/Cu_{var}, the accumulation appears to reach a limiting value at the highest Cu exposure concentration during the first days, which is less pronounced at day seven (SI-Fig 1 B and SI-Table 4). By the end of the experiment, Cu accumulation in both the experimental series in terms of percentage of increase was in the order gills > liver > carcass, whereas in terms of absolute values the order was liver > gills > carcass. In muscle and brain tissue, no statistically significant accumulation of Cu was observed for both exposures (SI-Table 5 and 6).

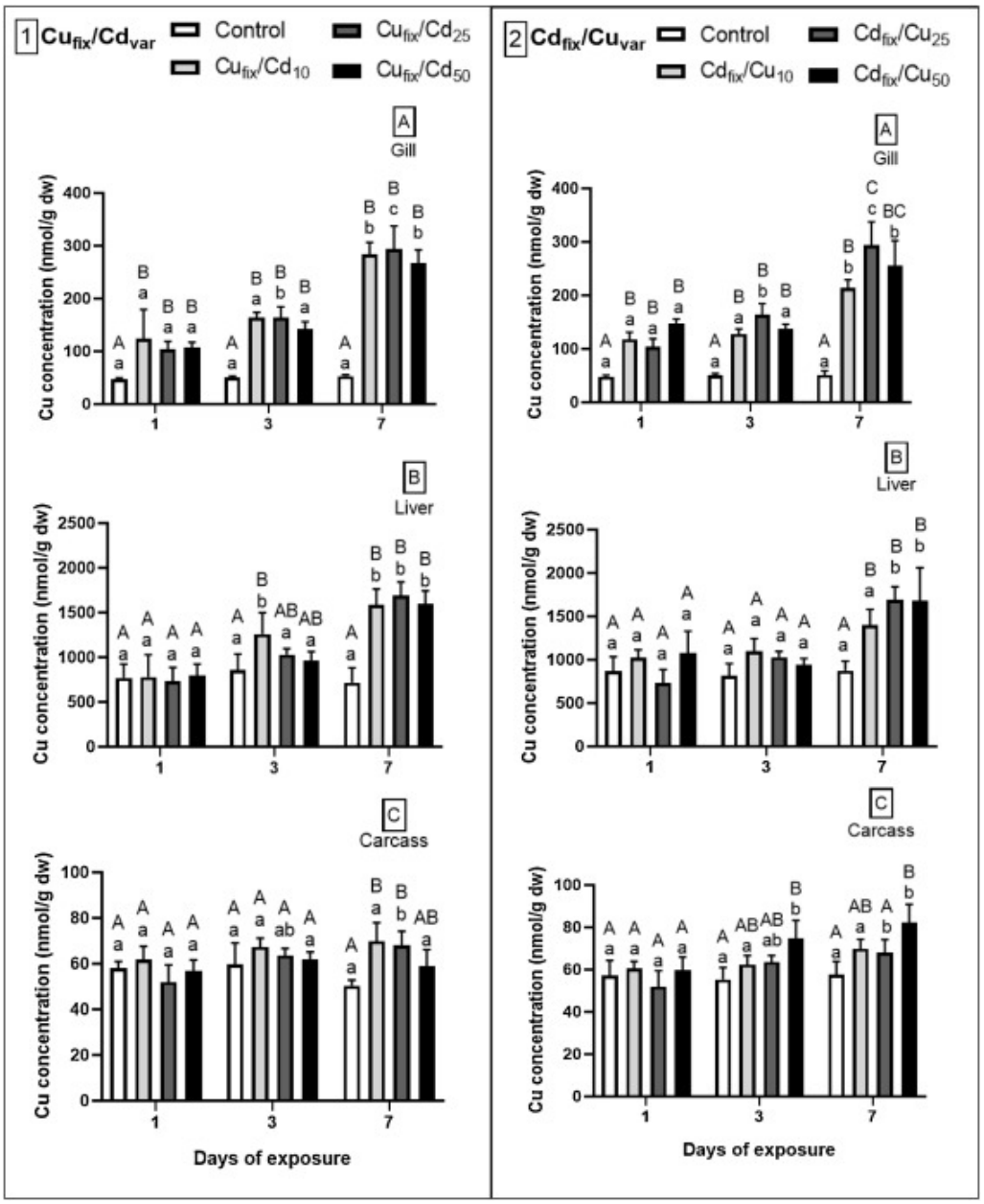


Fig. 1. Copper (Cu) concentration (nmol/g dry weight) in gills (A), liver (B) and carcass (C) of *Cyprinus carpio* exposed to Cu_{fix}/Cd_{var} (1) or Cd_{fix}/Cu_{var} (2) mixtures for 1, 3 and 7 days. Mean ± SD, N=5. Letters were only added when statistical differences occurred. Lower-case letters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

3.3.1.2. Cadmium bioaccumulation

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

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

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

Cadmium accumulation increased both through time and among the different exposure levels without reaching steady-state in the Cu_{fix}/Cd_{var} exposures (SI-Fig 2 A and B). Although metal concentrations increased with time in fish exposed to Cd_{fix}/Cu_{var} (SI-Fig 2 B), a clear dose dependent inhibition of Cu on Cd levels can be observed, with a fast reduction in Cd uptake at the highest Cu exposure level. The accumulation rates for Cd in fish exposed to $Cu_{fix}/Cd_{10\text{-}25\text{-}50}$ were $\simeq$ 0.3, 0.6 and 1/0.8 nmol g^{-1} dw h^{-1}, respectively for day one and three, whereas the accumulation rates dropped to 0.1, 0.3 and 0.4 nmol g^{-1} dw h^{-1}, respectively at the end of the experiment. In fish exposed to the $Cd_{fix}/Cu_{10\text{-}25\text{-}50}$ scenario, the accumulation rates expressed in nmol g^{-1} dw h^{-1}were 1.1, 0.6 and 0.4, respectively for day one and 0.9, 0.6 and 0.2, respectively at day three. A further decrease was observed at day seven were the accumulation rates were 0.5, 0.3 and 0.2 nmol g^{-1} dw h^{-1} respectively.

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

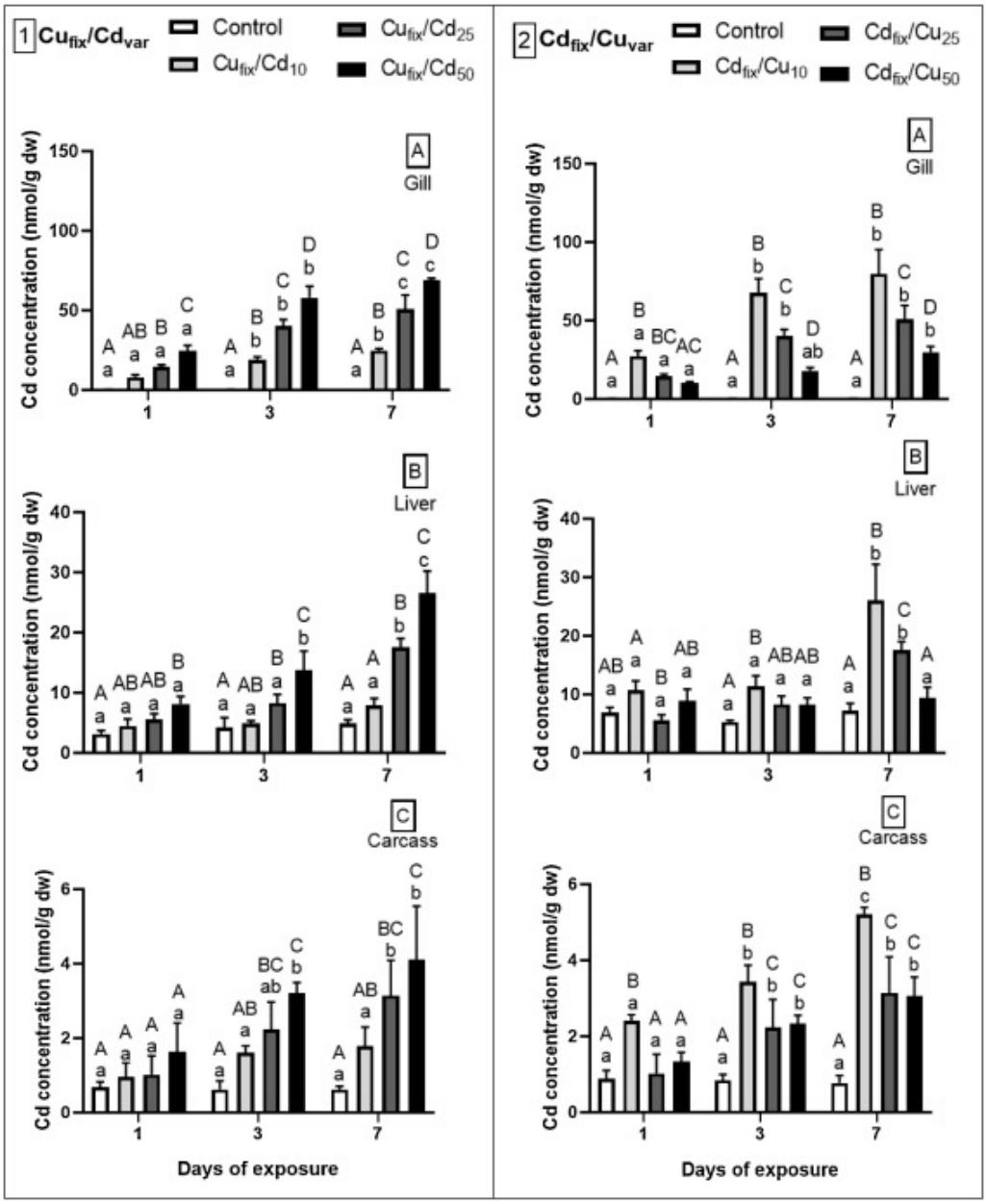


Fig. 2. Cadmium (Cd) concentration (nmol/g dry weight) in gills (A), liver (B) and carcass (C) of *Cyprinus carpio* exposed to Cu_{fix}/Cd_{var} (1) or Cd_{fix}/Cu_{var} (2) mixtures for 1, 3 and 7 days. Mean ± SD, N=5. Letters were only added when statistical differences occurred. Lower-case letters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

3.3.2. Expression of MTs and antioxidant enzymes

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

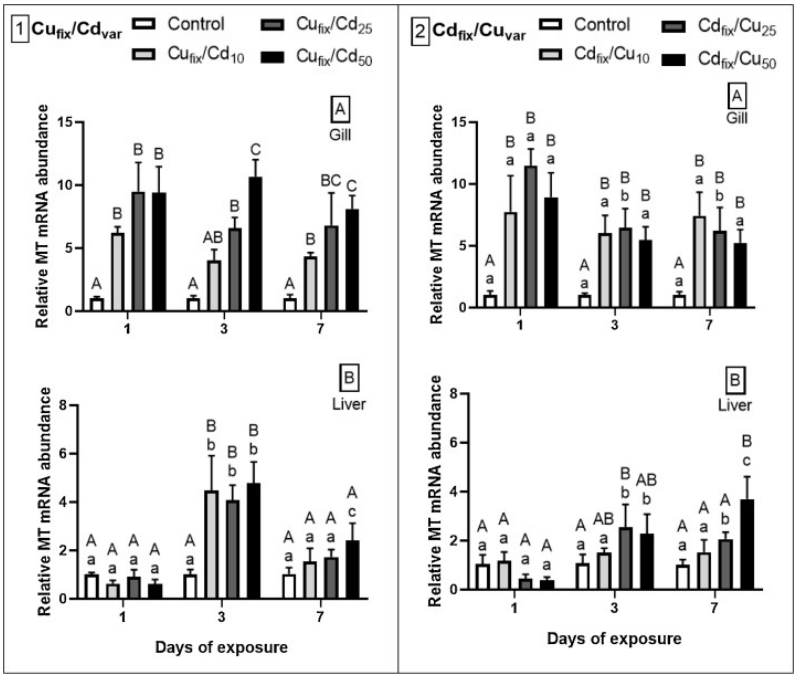


Fig. 3. Relative metallothionein (MT) mRNA abundance in gills (A) and liver (B) of *Cyprinus carpio* exposed to Cu_{fix}/Cd_{var} (1) or Cu_{var}/Cd_{fix} (2) mixtures for 1, 3 and 7 days. Mean ± SD, N=4. Letters were only added when statistical differences occurred. Lower-case letters indicate significant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

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

Regarding the expression of SOD in the gills, a significant increase in relative mRNA abundance compared to the control was observed from day three onwards in treatment Cu_{fix}/Cd_{10} and on day seven for treatment Cu_{fix}/Cd_{25} (Fig. 4.1B). During exposure Cd_{fix}/Cu_{var} a significant increase in relative SOD mRNA was observed after seven days in treatment Cd_{fix}/Cu_{25} and Cd_{fix}/Cu_{50} (Fig. 4.2B). Moreover, for both the exposure scenarios, no significant differences were observed among the treatments during the same sampling day. Furthermore, the gene expression of treatment Cd_{fix}/Cu_{50} at day seven is significantly higher compared with day one.
Concerning the expression of CAT in the liver, no significant differences were observed between the control and the treatment for fish exposed to Cu_{fix}/Cd_{var} (Fig. 4.1C). A significant decrease in relative mRNA abundance compared to the control was observed on day one and three for treatment Cd_{fix}/Cu_{50}; however, by day seven the levels were similar to the control (Fig. 4.2C).
Finally, for GST expression in the liver, a significant decrease in relative mRNA abundance compared to the control was observed for treatment Cu_{fix}/Cd_{10} after three days (Fig. 4.1D). During exposure Cd_{fix}/Cu_{var} (Fig. 4.2D), a significant increase compared to the control was observed in treatment Cd_{fix}/Cu_{25} after one day and a significant decrease in treatment Cd_{fix}/Cu_{50} after three days. The GST mRNA abundance significantly decreased compared to the first sampling day, in treatment Cd_{fix}/Cu_{10} and Cd_{fix}/Cu_{25} after three days (Fig. 4.2D).

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

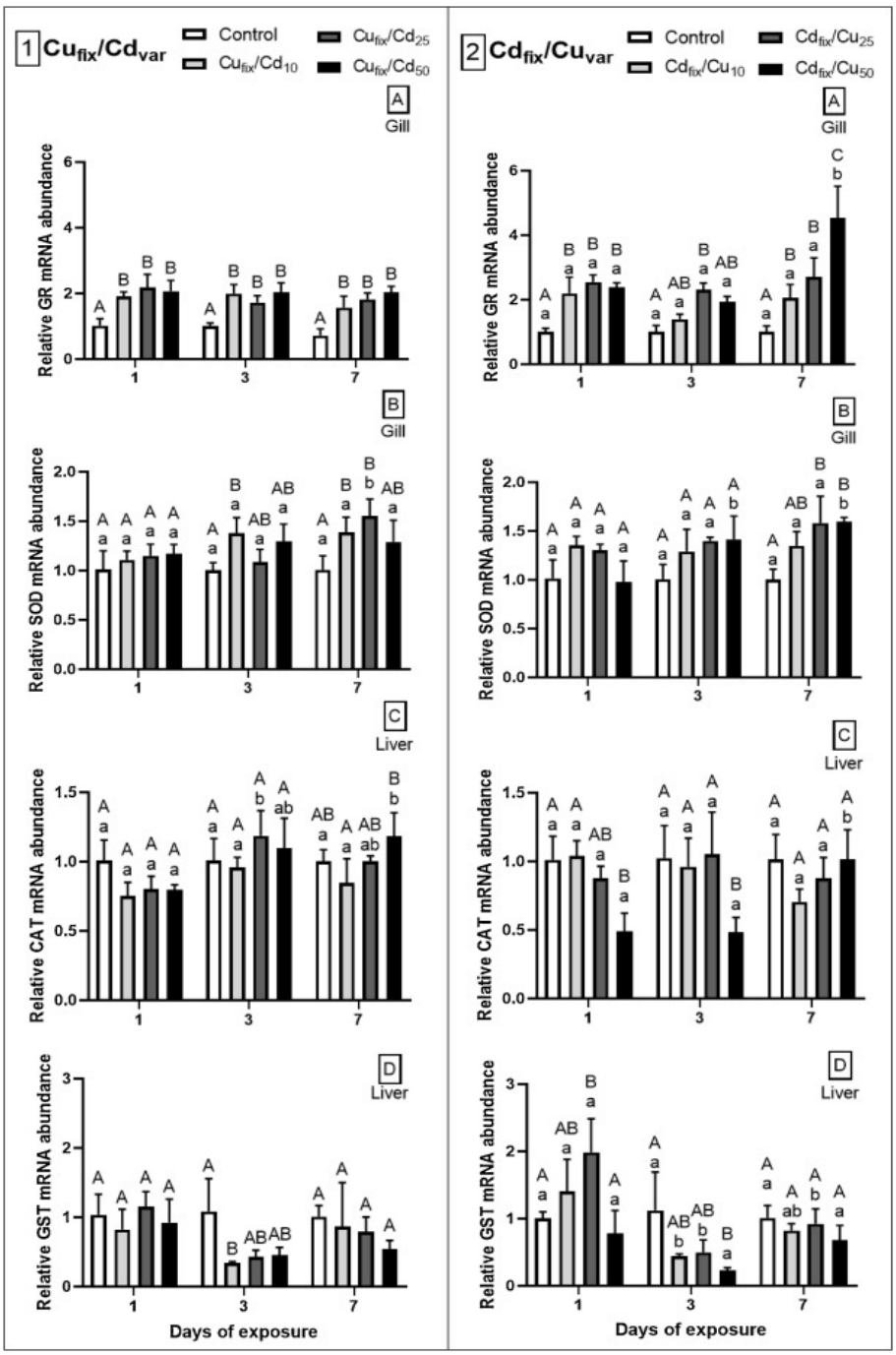


Fig. 4. Relative glutathione reductase (A), superoxide dismutase Cu-Zn (B), catalase (C) and glutathione S-transferase (D) mRNA abundance in gills and liver of *Cyprinus carpio* exposed to Cu_{fix}/Cd_{var} (1) or Cd_{fix}/Cu_{var} (2) mixtures for 1, 3 and 7 days. Mean ± SD, N=4. Letters were only added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

3.3.3. Effects of metal exposure on ionoregulation

3.3.3.1. Sodium

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

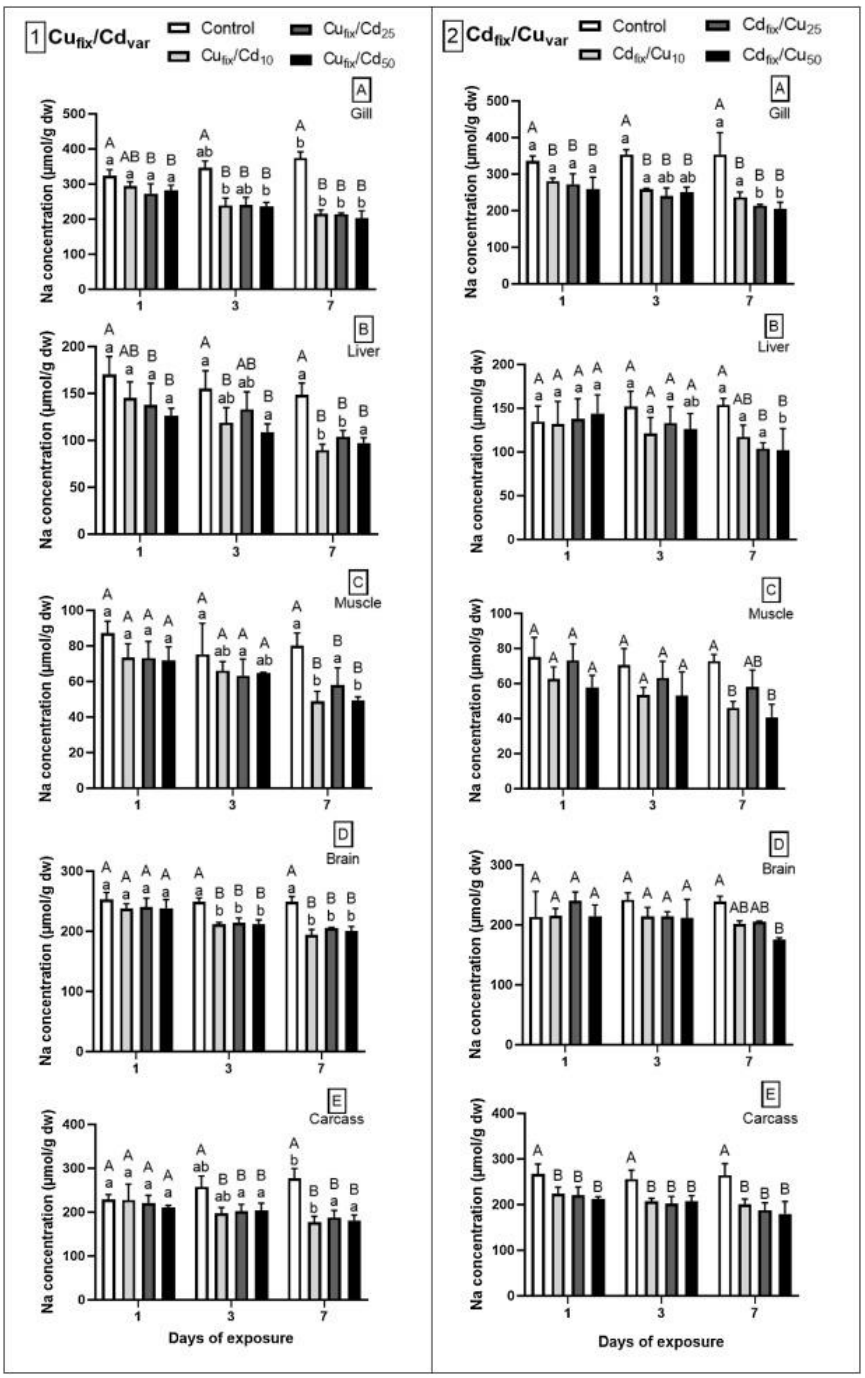


Fig. 5. Sodium (Na) concentration (µmol/g dry weight) in gills (A), liver (B), muscle (C), brain (D) and carcass (E) of *Cyprinus carpio* exposed to Cu_{fix}/Cd_{var} (1) or Cd_{fix}/Cu_{var} (2) mixtures for 1, 3 and 7 days. Mean ± SD, N=5. Letters were only added when statistical differences occurred.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

3.3.3.2. Potassium

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

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

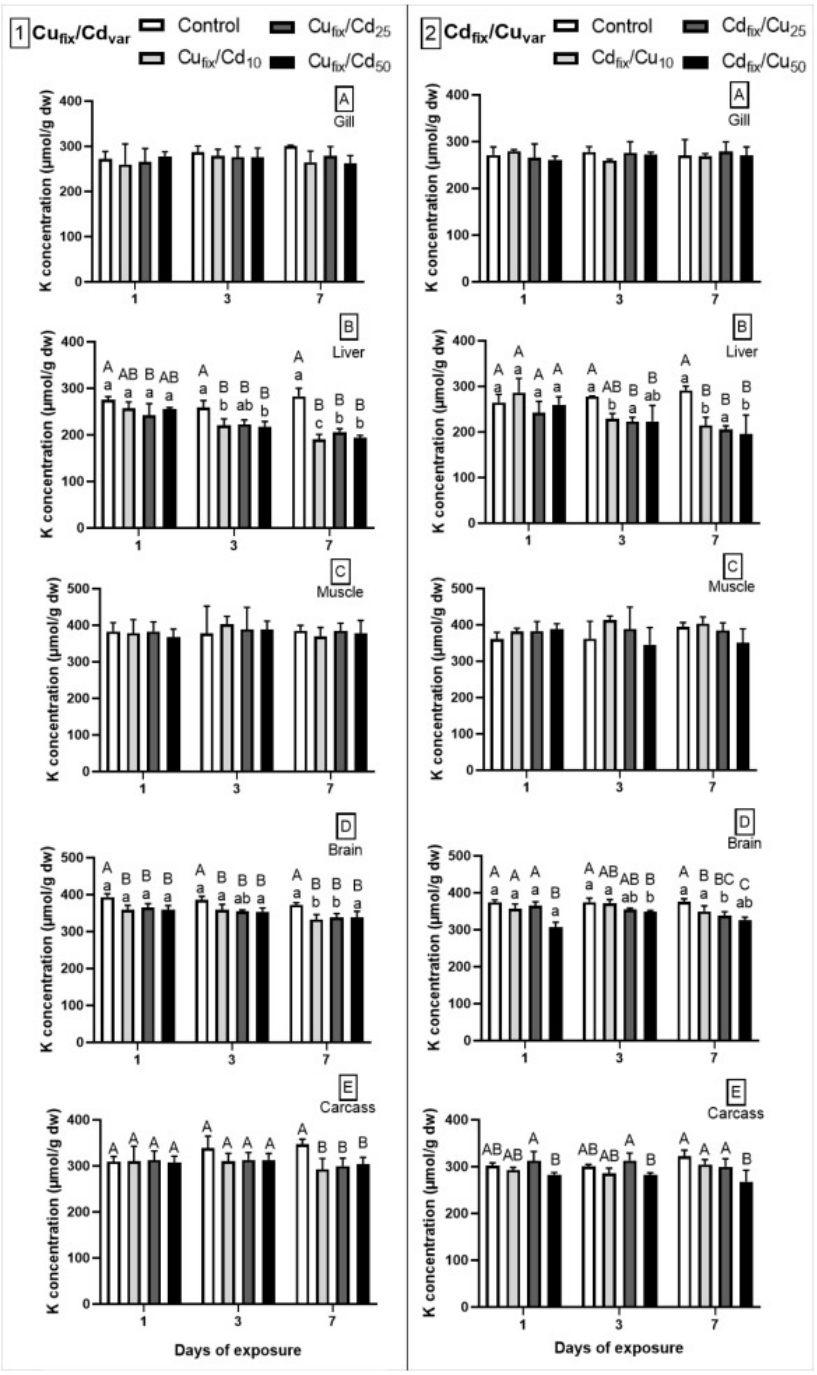


Fig. 6. Potassium (K) concentration (µmol/g dry weight) in liver (A), brain (B) and carcass (C) of *Cyprinus carpio* exposed to Cu_{fix}/Cd_{var} (1) or Cd_{fix}/Cu_{var} (2) mixtures for 1, 3 and 7 days. Mean ± SD, N=5. Letters were only added when statistical differences occurred. Lower-case letters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

3.3.3.3. Calcium and Magnesium

Calcium concentration in gills significantly decreased compared to the control after seven days in treatment Cu_{fix}/Cd_{50} (Fig. 7.A). For the liver, a significant decrease compared to the control was observed in treatment Cu_{fix}/Cd_{50} after three days, but not after seven days (Fig. 7.C).

Regarding the Mg content in the gills, no significant changes were observed during exposure Cu_{fix}/Cd_{var} (Fig. 7.B). For the liver, a significant decrease for all treatment groups compared to the control was evident after seven days (Fig. 7.D). Moreover, the Mg content declined significantly compared to day one, in treatment Cu_{fix}/Cd_{10} and Cu_{fix}/Cd_{50} at day three and treatment Cu_{fix}/Cd_{25} at day seven (Fig. 7.D). A further liver Mg concentration decrease compared to day three was observed in treatment Cu_{fix}/Cd_{10} after seven days (Fig. 7.D). For all other tissues, no significant changes in the Ca or Mg content were observed during both experimental series (SI-Table 5 and 6).

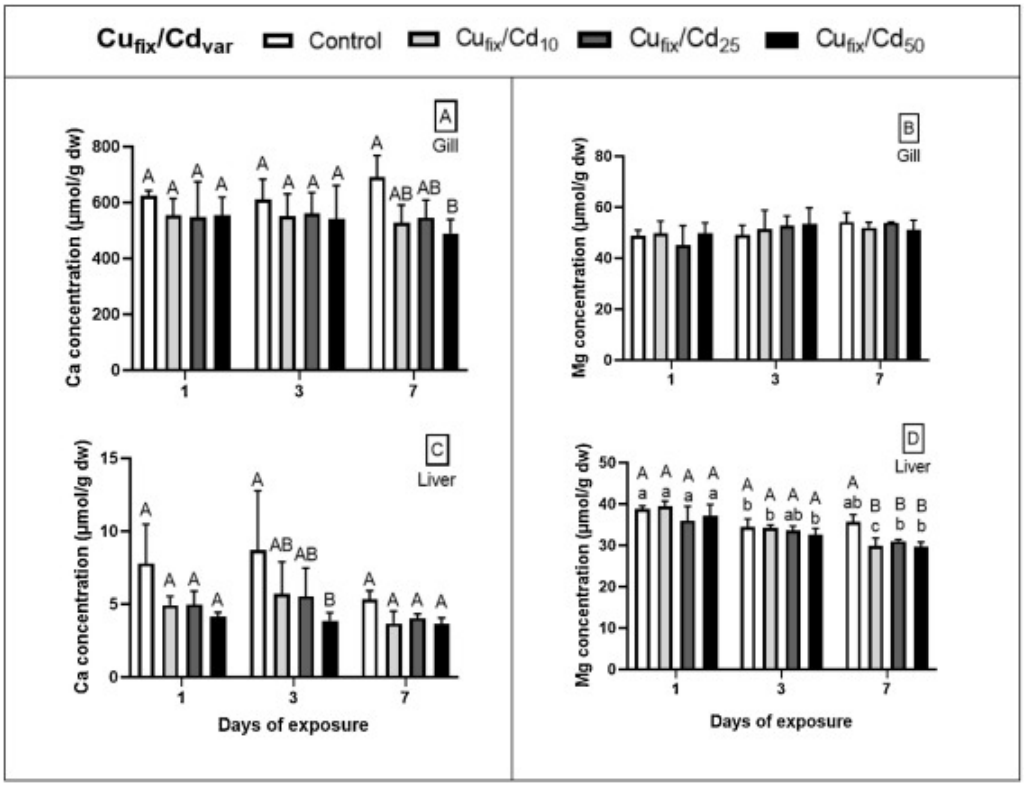


Fig. 7. Calcium and magnesium concentration (µmol/g dry weight) in gills (A,B) and liver (C,D) of *Cyprinus carpio* exposed to Cu_{fix}/Cd_{var} mixtures for 1, 3 and 7 days. Mean ± SD, N=5. Letters were only added when statistical differences occurred. Lower-case letters indicate significant differences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

3.3.3.4. Gene expression of ion channels in the gills

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

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

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

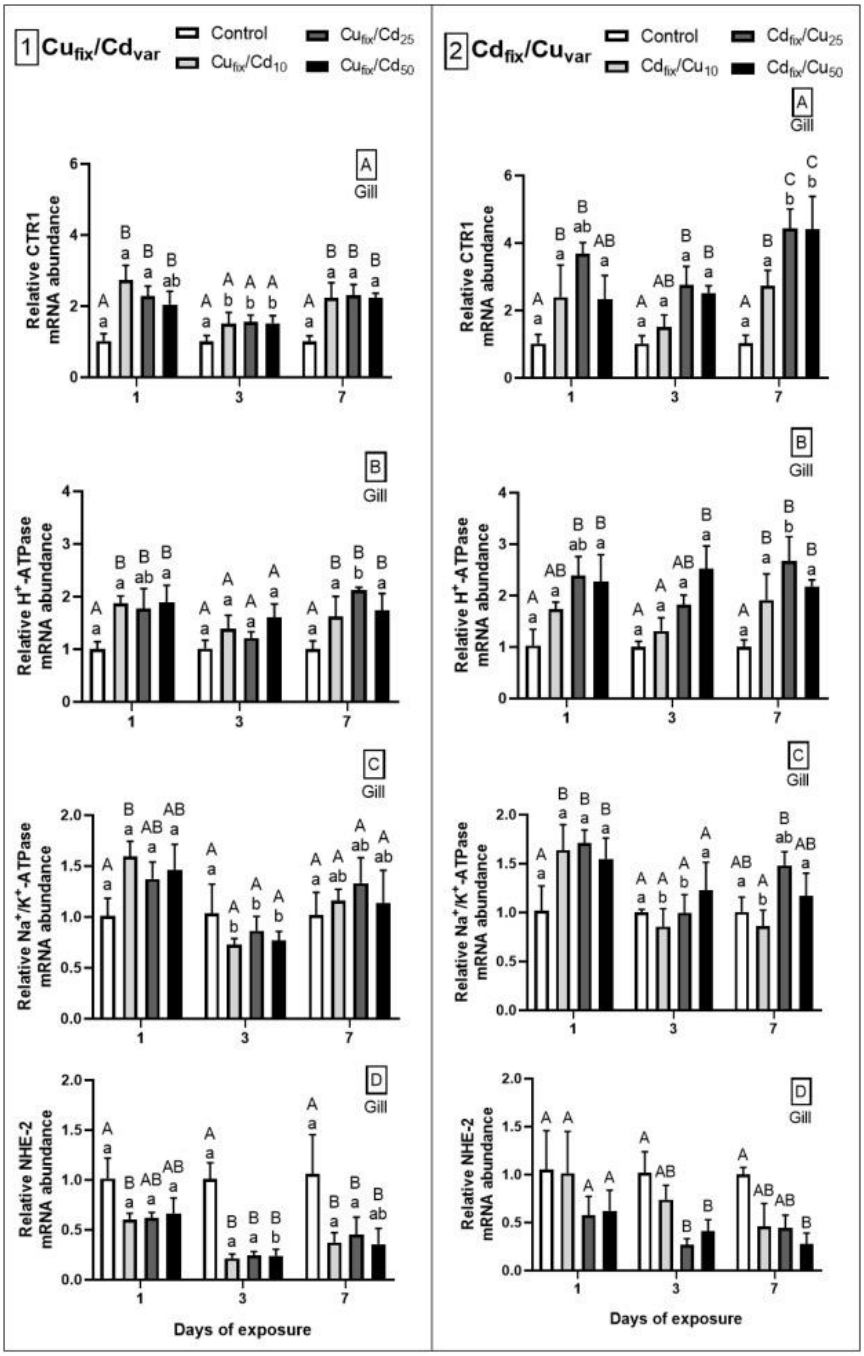


Fig. 8. Relative copper transporter 1 (A), H^+-ATPase (B), Na^+/K^+-ATPase (C) and Na^+/H^+-exchanger (D) mRNA abundance in gills of *Cyprinus carpio* exposed to to Cu_{fix}/Cd_{var} (1) or Cd_{fix}/Cu_{var} (2) mixtures for 1, 3 and 7 days. Mean ± SD, N=4. Letters were only added when statistical differences occurred. Lower-case letters indicate significant differences ($p < 0.05$) of treatments among sampling days, capital letters indicate significant differences ($p < 0.05$) among treatments within the same sampling day.

3.4. Discussion

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

3.4.1. Dynamics of Cu and Cd bioaccumulation

3.4.1.1. Copper bioaccumulation

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

When comparing the results obtained in the single exposure scenario using comparable metal concentrations, in which the net accumulated values after one week were approximately 112, 182 and 308 nmol/g dw for Cu, and 81, 137 and 267 nmol/g dw for Cd (Castaldo et al. 2020a, Castaldo et al. 2020c) with results obtained in the binary mixture, it can be noticed that Cu content is slightly higher in the treatments $Cd_{fix}/Cu_{10\text{-}25}$ (respectively ≃ 44-32 %) but that is not for the case of exposure to Cd_{fix}/Cu_{50}. Also in the Cu_{fix}/Cd_{var} exposure series, Cu accumulated to values slightly higher than those found for 25% of the 96 h-LC_{50} in the single Cu exposures (≃ 17-32%). This confirms that there was no systematic inhibiting effect of Cd on Cu accumulation, if anything, it was slightly stimulating (except at the highest Cu exposure). Results on Cu accumulation inhibition/stimulation by Cd are often inconsistent. A reduction of Cu uptake in presence of Cd was demonstrated in water flea (*Daphnia magna*) and in rainbow trout (Komjarova and Blust 2008, Niyogi et al. 2015). However, in another study, in which zebrafish were exposed to an increasing concentration of Cd (0.01, 0.05, 0.2 µM) plus a fixed concentration of Cu (0.02 µM), the presence of Cd did not alter Cu uptake (Komjarova and Blust 2009). Moreover, in rainbow trout a stimulation of Cu uptake in presence of Cd occurred (Brix et al. 2016). Therefore, the explanation of shared transporters and non-specific competition for

binding sites seems reasonable (Niyogi et al. 2015). In fact, even though Cd and Cu are considered to be respectively the Ca^{2+} and Na^{+} antagonists (Grosell and Wood 2002, Niyogi and Wood 2004), several studies provided evidence of shared uptake routes of these metals in fish gills via the ECaC, DMT1 and Zip-8 (Cooper et al. 2007, Alsop and Wood 2011, Komjarova and Bury 2014, Niyogi et al. 2015). Alternatively, effects might go unnoticed, since after the first day with faster accumulation rates, the Cu accumulation rate seems to attain a steady-state value which is similar over all exposure conditions.

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

Several mechanisms in vertebrates are known to play a role in Cu homeostasis, such as the CTR1 and the Cu-ATPase (Anni et al. 2019). The CTR1 has been proposed as a Cu^{+} transporter which is insensitive to external Na^{+} concentrations (Mackenzie et al. 2004, Craig et al. 2010, Komjarova and Bury 2014). According to Grosell and Wood (2002) the copper uptake pathway which is sensitive to external Na^{+} concentration dominates in environments with a Na^{+} deficiency, whereas the Na^{+} insensitive copper

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

3.4.1.2. Cadmium bioaccumulation

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

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

the gills (Vinodhini and Narayanan 2008) and considering the very low background levels, accumulation was significant from the beginning, with the exception of fish exposed to Cd_{fix}/Cu_{50}.

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

The rapid and substantial Cd accumulation in the gills reflects their role as the primary uptake site of metal ions during waterborne exposures, and indicates the vulnerability of these tissues (Benhamed et al. 2016). The rapid Cd accumulation, similar to Cu, is consistent with the very high affinity that Cd has for gill binding sites (conditional log *K* of 8.6 (dm^3 mol^{-1}), calculated at pH 6.2 and ionic strength of 1.e-04)(Playle et al. 1993, Playle 2004) and the capacity thereof, together with the non-complexing nature of our exposure media. Considering the binding constants, one can assume that relatively more Cd, rather than Cu, should bind to the gill surface. However, our results showed a higher Cu-compared to Cd accumulation. Therefore, we can hypothesize that both metals entered the cell, but Cu displaced Cd from MTs due to a higher affinity for the protein (Vašák 1991). Thus, Cd will be subsequently flushed away into the kidney for excretion processes, whereas Cu will remain into the tissue bound to MTs. Moreover, we can also consider firstly, that an antagonistic-like effect, of Cu on Cd uptake due to the shared branchial uptake routes will occur (Alsop and Wood 2011, Komjarova and Bury 2014), secondly that at the same equitoxic concentrations, Cd levels were almost three times lower compared to Cu, and thirdly that our fish were fasted. In fact a higher Cu accumulation was reported in fish exposed to a reduced food ratio(Hashemi et al. 2008a). Therefore, we hypothesize that due to the absence of food, fish tried to compensate for the electrolyte losses, by enhancing the uptake of essential elements from the water, thereby promoting Cu uptake. Moreover, it has been suggested that Cu binding capacity (B_{max}) can vary reflecting changing requirements of this essential metal in growing juvenile fish (Brix et al. 2016).

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

3.4.2. Defensive mechanisms

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

As already mentioned, in addition to MTs, various antioxidant enzymes are present in cells to cope with deleterious effects caused by ROS(Wang et al. 2010). In the present work, we investigated the relative gene expression of SOD, CAT, GR and GST both in the liver and in the gills. The results obtained in the gills for both the exposure series are quite similar. Glutathione reductase is an enzyme involved in the renewal of GSH, and together with GST, it plays a key role in the GSSG/GSH balance (Dautremepuits et

al. 2009). We observed an upregulation of the GR gene, similar to what was reported for sea bream exposed to Cu (Minghetti et al. 2008) and obscure pufferfish (*Takifugu obscurus*) exposed to Cd(Kim et al. 2010). Moreover,Kim et al. (2010), proposed that GR is one of the main antioxidants against Cd-induced oxidative stress in *obscure pufferfish*, since its transcript had the highest expression level in all examined tissues. It is known that common carp rely on GSH, which can bind metal ions, as a first line of defence(Eyckmans et al. 2011). Therefore, an increase in the GR gene expression is expected to reduce the level of oxidized glutathione. The obtained results, similar to what observed in a previous study (Castaldo et al. 2020c), suggested that a saturation of base levels of MT and GSH occurred, due to their ability to bind metal ions (Lange et al. 2002), and that a continuous production of the proteins is needed to handle the increasing amount of metals in the gills.

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

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

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

3.4.3. Changes in electrolyte levels and organism responses

Metal ions are known to interfere with electrolyte ion homeostasis, due to competition at the uptake sites (Čelechovská et al. 2007, Niyogi et al. 2015). Among the electrolytes, Na^+ is the major cation of the extracellular fluid (Sathya et al. 2012). In

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

As reported in previous studies, and in different species such as Nile tilapia and rainbow trout, gills are the most affected tissue in waterborne exposures (Grosell and Wood 2002, Mackenzie et al. 2004, Atli and Canli 2011, Niyogi et al. 2015). A drastic loss in total Na can result in death, however different species can tolerate different percentages of loss. For example the threshold for rainbow trout and yellow perch is set to 30 % and 40 % loss of whole body electrolyte content, respectively (Taylor et al. 2003), whereas gibel carp can tolerate losing up to the 45% of their whole body total Na content (De Boeck et al. 2010b). In the present study the gill loss of total Na at day seven ranged between 33 and 45 %, whereas in carcass the Na decrease ranged between 24 and 36 %. However, Na levels in the gill of control animals slightly increased from day one to seven (approximately 15%) which explains the high percentage (45 %) of Na loss encountered in fish gills exposed to Cu_{fix}/Cd_{var}.

Sodium can enter the gills through a putative Na^+-channel powered by H^+-ATPase, a Na^+/Cl^- cotransporter and the NHEs (McCormick 2001, Kumai and Perry 2012). In order to understand mechanisms that act to maintain Na^+-homeostasis, we analysed the expression of genes coding for Na^+/K^+-ATPase, H^+-ATPase and the NHE-2 in the gills. An upregulation of the Na^+/K^+-ATPase gene expression was recorded in fish exposed to Cd_{fix}/Cu_{var} after one day for all the treatments compared to the control. In agreement withBoyle et al. (2011), we hypothesize that the increase in the Na^+/K^+-ATPase gene expression could be a compensatory mechanism to counteract the inhibition of the enzyme activity, caused by metals as already reported by several authors (De Boeck et al. 2001, Eyckmans et al. 2011) for the inhibition of the enzyme activity.
The active uptake of Na^+ in freshwater fish is necessary for ionic homeostasis, and as already mentioned, Na^+ can be taken up via a Na^+/H^+ exchanger or via a putative Na^+-channel coupled with H^+-ATPase (Wilson et al. 2000). Several NHEs have been identified and NHE-2 has been proposed as a candidate for the Na^+-sensitive component of Cu^+ uptake (Mackenzie et al. 2004, Craig et al. 2010, Komjarova and Bury 2014). In contrast with the observations reported byKomjarova and Bury (2014), our results showed a decreasing trend in NHE-2 mRNA abundance in both the exposure

series. If NHE-2 can be proposed as a Cu uptake component, this reduction can be interpreted as an attempt by the fish to reduce the direct Cu uptake, and/or as a way to cut down the extrusion of protons in order to minimize the influx of Cu. Nevertheless, the latter assumption is unlikely as the H^+-ATPase expression was upregulated. Alternatively, as described by Grosell (2011), it can be an indirect effect caused by the inhibition of the carbonic anhydrase, which leads to a depletion of the necessary substrate for the exchanger. Theoretically, the lower amount of substrate could lead to a reduced efficiency of the Na^+/H^+- exchanger but not of the H^+-ATPase which is an active transporter. However, in both cases one of the outcomes is a drop of internal Na^+ content, which the organisms try to compensate for by increasing the expression of the gene coding for H^+-ATPase. Nonetheless, the Na^+ loss in the gills has repercussions on the whole-body Na levels.

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

According to previous results obtained in our lab with common carp (Delahaut et al. 2020) we did not expect a loss in total Ca. Nonetheless, after one week of exposure in fish exposed to Cu_{fix}/Cd_{50} an electrolyte decrease occurred in the gills, suggesting a synergistic-like effect between metal ions on Ca loss. For the liver, a significant Ca decrease was observed for treatment Cu_{fix}/Cd_{50} after three days, but not after seven days. This trend is likely due to the considerable variation in the Ca concentration of the liver in fish from the control group throughout the exposure. A competition between Cd^{2+} and Ca^{2+} at the apical and basolateral Ca^{2+}-channel has been reported resulting in hypocalcaemia (McGeer et al. 2000b, Niyogi et al. 2015). The significant decrease observed only after one week indicates that both time and the exposure concentrations play a crucial role. Moreover, one can assume that the presence of Cu contributed to this loss. Considering that previous studies on zebrafish showed that Cu decreases Ca^{2+} uptake and vice versa (Craig et al. 2010, Alsop and Wood 2011), we hypothesize that a competition between Ca^{2+} and Cu^{2+} at the uptake site occurred (Grosell 2011). Moreover, it has been suggested that Cu^{2+} epithelial transport may be via a Ca^{2+} pathway (Alsop and Wood 2011, Komjarova and Bury 2014).

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

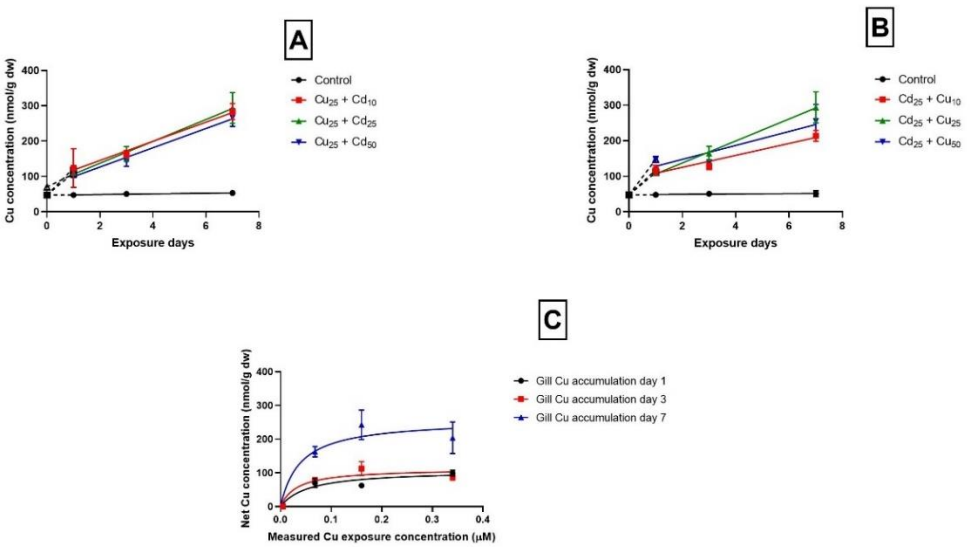
3.5. Conclusions

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

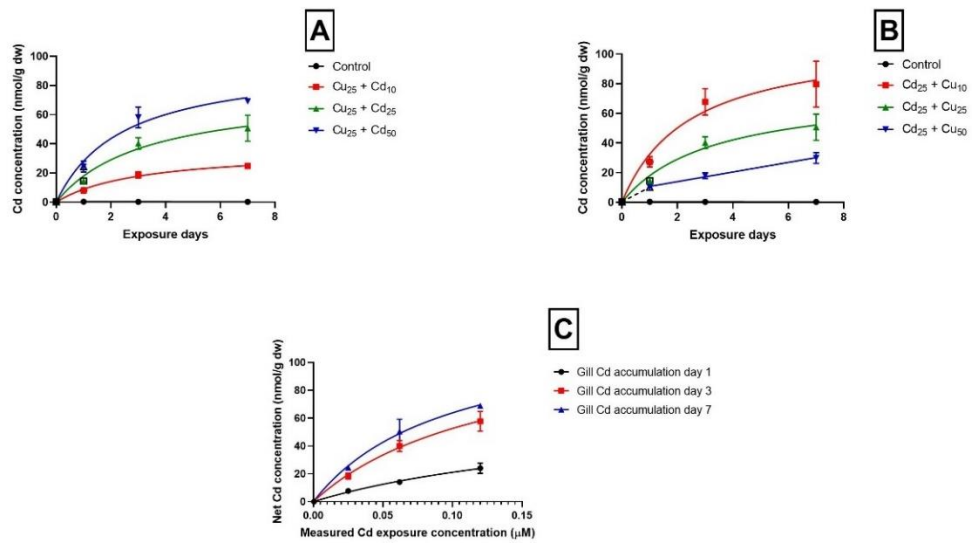
Acknowledgments

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

3.6. Supplementary information



SI - Fig 1: Cu concentration over time in both the Cu_{fix}/Cd_{var} (A) and Cd_{fix}/Cu_{var} (B) exposures, dotted lines indicate assumed accumulation rates during the first day, extrapolating starting values from control values at day 1; (C) net Cu concentration as function of the concentration in the Cd_{fix}/Cu_{var} exposure. The Michaelis-Menten (Y = Vmax*X/(Km + X)) curves (C) were fitted using measured concentration of the variable metal of each experimental series.



SI - Fig 2: (A) Cd concentration over time in Cu_{fix}/Cd_{var} (A) and Cd_{fix}/Cu_{var} (B) exposures, dotted lines indicate assumed accumulation rates during the first day, extrapolating starting values from control values at day 1; (C) net Cd concentration as function of the concentration in Cu_{fix}/Cd_{var} exposure. The Michaelis-Menten (Y = Vmax*X/(Km + X)) curves were fitted using measured concentration of the variable metal of each experimental series.

SI – Table 1*:* Chemical speciation in the mixture of the Cd_{fix}/Cu_{var} exposure, calculated using measured concentrations with the equilibrium speciation code VMinteq.

Component	**Concentration (mol/l)**	**% of total concentration**	**Species name**
(10% 96 h LC_{50}) Cu^{2+}	1.8771E-09	2.7	Cu^{2+}
		8.8	$CuOH^{+}$
		0.02	$Cu(OH)_3^{-}$
		1.6	$Cu(OH)_2$ (aq)
		0.2	$CuSO_4$ (aq)
		84.3	$CuCO_3$ (aq)
		0.1	$CuHCO_3^{+}$
		2.0	$Cu(CO3)_2^{2-}$
(25% 96 h LC_{50}) Cu^{2+}	4.4169E-08	2.7	Cu^{2+}
		8.8	$CuOH^{+}$
		0.02	$Cu(OH)_3^{-}$
		1.6	$Cu(OH)_2$ (aq)
		0.2	$CuSO_4$ (aq)
		84.3	$CuCO_3$ (aq)
		0.1	$CuHCO_3^{+}$
		2.0	$Cu(CO3)_2^{2-}$
(50% 96 h LC_{50}) Cu^{2+}	9.3860E-09	2.7	Cu^{2+}
		8.8	$CuOH^{+}$
		0.02	$Cu(OH)_3^{-}$
		1.6	$Cu(OH)_2$ (aq)
		0.02	$CuSO_4$ (aq)
		0.2	$CuCO_3$ (aq)
		84.3	$CuHCO_3^{+}$
		0.1	$Cu(CO3)_2^{2-}$
(25% 96 h LC_{50}) Cd^{2+}	4.7846E-08	79.7	Cd^{2+}
		0.5	$CdOH^{+}$
		0.3	$CdCl^{+}$
		7.6	$CdSO_4$ (aq)
		0.08	$Cd(SO_4)_2^{2-}$
		1.9	$CdHCO_3^{+}$
		9.7	$CdCO_3$ (aq)
		0.06	$Cd(CO_3)_2^{2-}$

SI – Table 2: Chemical speciation in the mixture of the Cu_{fix}/Cd_{var} exposure, calculated using measured concentrations with the equlibrium speciation code VMinteq.

Component	**Concentration (mol/l)**	**% of total concentration**	**Species name**
(10% 96 h LC_{50}) Cd^{2+}	1.9936E -08	79.7	Cd^{2+}
		0.5	$CdOH^{+}$
		0.3	$CdCl^{+}$
		7.6	$CdSO_4$ (aq)
		0.08	$Cd(SO_4)_2^{2-}$
		1.9	$CdHCO_3^{+}$
		9.7	$CdCO_3$ (aq)
		0.06	$Cd(CO_3)_2^{2-}$
(25% 96 h LC_{50}) Cd^{2+}	4.9441E-08	79.7	Cd^{2+}
		0.5	$CdOH^{+}$
		0.3	$CdCl^{+}$
		7.6	$CdSO_4$ (aq)
		0.08	$Cd(SO_4)_2^{2-}$
		1.9	$CdHCO_3^{+}$
		9.7	$CdCO_3$ (aq)
		0.06	$Cd(CO_3)_2^{2-}$
(50% 96 h LC_{50}) Cd^{2+}	9.5692E-07	79.7	Cd^{2+}
		0.5	$CdOH^{+}$
		0.3	$CdCl^{+}$
		7.6	$CdSO_4$ (aq)
		0.08	$Cd(SO_4)_2^{2-}$
		1.9	$CdHCO_3^{+}$
		9.7	$CdCO_3$ (aq)
		0.06	$Cd(CO_3)_2^{2-}$
(25% 96 h LC_{50}) Cu^{2+}	1.0256E-08	2.7	Cu^{2+}
		8.8	$CuOH^{+}$
		0.02	$Cu(OH)_3^{-}$
		1.6	$Cu(OH)_2$ (aq)
		0.2	$CuSO_4$ (aq)
		84.3	$CuCO_3$ (aq)
		0.1	$CuHCO_3^{+}$
		2.0	$Cu(CO3)_2^{2-}$

SI - Table 3: Metal content, metal net accumulation, percentage of increase and accumulation rate in the gills of common carp exposed to the binary mixture Cu_{fix}/Cd_{var} for 1, 3 and 7 days.

Cu_{fix}/Cd_{var} Cu Content												
	Day 1				Day 3				Day 7			
Exposure	**Control**	**Cu_{25}/Cd_{10}**	**Cu_{25}/Cd_{25}**	**Cu_{25}/Cd_{50}**	**Control**	**Cu_{25}/Cd_{10}**	**Cu_{25}/Cd_{25}**	**Cu_{25}/Cd_{50}**	**Control**	**Cu_{25}/Cd_{10}**	**Cu_{25}/Cd_{25}**	**Cu_{25}/Cd_{50}**
Total metal content (nmol/g dw)	48 ± 2	124 ± 55	104 ± 15	107 ± 10	51 ± 2	164 ± 10	164 ± 20	143 ± 13	53 ± 2	284 ± 22	294 ± 43	267 ± 25
Net accumulation (nmol/g dw)	/	76 ± 55	56 ± 15	59 ± 10	/	113 ± 10	113 ± 20	92 ± 13	/	230 ± 22	240 ± 43	213 ± 25
% increase	/	158 ± 116	118 ± 32	125 ± 21	/	223 ± 19	223 ± 40	182 ± 27	/	433 ± 42	452 ± 82	401 ± 47
Accumulation rate (nmol g-1 dw h-1)	/	3.2 ± 2	2.3 ± 0.6	2.5 ± 0.4	/	1.6 ± 0.1	1.6 ± 0.3	1.3 ± 0.2	/	1.4 ± 0.1	1.4 ± 0.3	1.3 ± 0.2
Cu_{fix}/Cd_{var} Cd Content												
	Day 1				Day 3				Day 7			
Exposure	**Control**	**Cu_{25}/Cd_{10}**	**Cu_{25}/Cd_{25}**	**Cu_{25}/Cd_{50}**	**Control**	**Cu_{25}/Cd_{10}**	**Cu_{25}/Cd_{25}**	**Cu_{25}/Cd_{50}**	**Control**	**Cu_{25}/Cd_{10}**	**Cu_{25}/Cd_{25}**	**Cu_{25}/Cd_{50}**
Total metal content (nmol/g dw)	0.4± 0.04	8 ± 1	15 ± 1	24 ± 3	0.32 ± 0.12	19 ± 2	40 ± 4	58 ± 7	0.3 ± 0.03	25 ± 1	50 ± 9	69 ± 1
Net accumulation (nmol/g dw)	/	7 ± 1	14 ± 1	24 ± 3	/	18 ± 2	40 ± 4	57 ± 7	/	24 ± 1	50 ± 9	69 ± 1
% increase	/	1919 ± 362	3543 ± 329	6011 ± 917	/	5859 ± 678	12689 ± 1262	18327 ± 2250	/	7979 ± 441	16448 ± 2918	22485 ± 319
Accumulation rate (nmol g-1 dw h-1)	/	0.3 ± 0.1	0.6 ± 0.1	1 ± 0.2	/	0.3 ± 0	0.6 ± 0.1	0.8 ± 0.1	/	0.1 ± 0.2 0	0.3 ± 0.1	0.4 ± 0

SI - Table 4: Metal content, metal net accumulation, percentage of increase and accumulation rate in the gills of common carp exposed to the binary mixture Cd_{fix}/Cu_{var} for 1, 3 and 7 days.

Cd_{fix}/Cu_{var} Cu Content												
	Day 1				Day 3				Day 7			
Exposure	**Control**	**Cd_{25}/Cu_{10}**	**Cd_{25}/Cu_{25}**	**Cd_{25}/Cu_{50}**	**Control**	**Cd_{25}/Cu_{10}**	**Cd_{25}/Cu_{25}**	**Cd_{25}/Cu_{50}**	**Control**	**Cd_{25}/Cu_{10}**	**Cd_{25}/Cu_{25}**	**Cd_{25}/Cu_{50}**
Total metal content (nmol/g dw)	48 ± 2	118 ± 13	104 ± 15	148 ± 8	51 ± 2	128 ± 9	164 ± 20	138 ± 8	51 ± 7	213 ± 15	294 ± 43	255 ± 46
Net accumulation (nmol/g dw)	/	70 ± 13	56 ± 15	99 ± 8	/	77 ± 9	112 ± 20	87 ± 8	/	162 ± 15	242 ± 43	204 ± 46
% increase	/	145 ± 27	116 ± 32	207 ± 17	/	151 ± 18	221 ± 40	171 ± 16	/	317 ± 30	473 ± 85	398 ± 91
Accumulation rate (nmol g-1 dw h-1)	/	3 ± 0.5	2.3 ± 0.3	4 ± 0.3	/	1 ± 0.1	1.6 ± 0.3	1.2 ± 0.1	/	1 ± 0.1	1.4 ± 0.3	1.2 ± 0.3
Cd_{fix}/Cu_{var} Cd Content												
	Day 1				Day 3				Day 7			
Exposure	**Control**	**Cd_{25}/Cu_{10}**	**Cd_{25}/Cu_{25}**	**Cd_{25}/Cu_{50}**	**Control**	**Cd_{25}/Cu_{10}**	**Cd_{25}/Cu_{25}**	**Cd_{25}/Cu_{50}**	**Control**	**Cd_{25}/Cu_{10}**	**Cd_{25}/Cu_{25}**	**Cd_{25}/Cu_{50}**
Total metal content (nmol/g dw)	0.38 ± 0.08	27 ± 3	15 ± 1	10 ± 1	0.32 ± 0.05	68 ± 9	40 ± 4	18 ± 2	0.32 ± 0.05	80 ± 15	51 ± 8	30 ± 3
Net accumulation (nmol/g dw)	/	27 ± 3	14 ± 1	10 ± 1	/	67 ± 9	40 ± 4	17 ± 2	/	79 ± 15	50 ± 9	29 ± 3
% increase	/	7134 ± 907	3705 ± 348	2595 ± 235	/	20970 ± 2789	12435 ± 1237	5499 ± 629	/	24910 ± 4857	15800 ± 2803	9285 ± 1130
Accumulation rate (nmol g-1 dw h-1)	/	1.1 ± 0.1	06 ± 0.1	0.4 ± 0	/	0.9 ± 0.1	0.6 ± 0.1	0.2 ± 0	/	0.5 ± 0.1	0.3 ± 0.1	0.2 ± 0

SI - Table 5: Metal concentration (nmol/g dw) and electrolyte levels (µmol/g dw) in different tissues of *Cyprinus carpio* exposed to Cu_{fix}/Cd_{var} mixtures for 1, 3 and 7 days. Mean ± SD, N=5. Lower-case letters in subscript indicate significant differences (p<0.05) of treatments among sampling days, capital letters in superscript indicate significant differences (p <0.05) among treatments within the same sampling day.

	Day 1				Day 3				Day 7			
Gill	**Control**	**Cu_{fix}/Cd_{10}**	**Cu_{fix}/Cd_{25}**	**Cu_{fix}/Cd_{50}**	**Control**	**Cu_{fix}/Cd_{10}**	**Cu_{fix}/Cd_{25}**	**Cu_{fix}/Cd_{50}**	**Control**	**Cu_{fix}/Cd_{10}**	**Cu_{fix}/Cd_{25}**	**Cu_{fix}/Cd_{50}**
Cu	48 ± 2.1^{A}	$124 \pm 49^{B}_{a}$	$104 \pm 14^{B}_{a}$	$107 \pm 9.2^{B}_{a}$	50.6 ± 2.2^{A}	$163.7 \pm 8.9^{B}_{a}$	$164 \pm 19^{B}_{b}$	$143 \pm 12^{B}_{a}$	53 ± 2^{A}	$284 \pm 20^{B}_{b}$	$294 \pm 39^{B}_{c}$	$267 \pm 23^{B}_{b}$
Cd	0.4 ± 0.051^{A}	$8.1 \pm 1.4^{AB}_{a}$	$15 \pm 1.3^{B}_{a}$	$24 \pm 3.7^{C}_{a}$	0.32 ± 0.14^{A}	$19 \pm 2.1^{B}_{b}$	$40 \pm 4.0^{C}_{b}$	$58 \pm 7.1^{D}_{b}$	0.31 ± 0.043^{A}	$25 \pm 1.4^{B}_{b}$	$51 \pm 8.9^{C}_{c}$	$69 \pm 0.98^{D}_{c}$
Ca	625 ± 18	554 ± 60	548 ± 126	554 ± 65	610 ± 74	551 ± 80	560 ± 76	542 ± 119	690± 78^{A}	528 ± 63^{AB}	545 ± 64^{AB}	489 ± 50^{B}
K	273 ± 16	260 ± 45	266 ± 29	277 ± 10	288 ± 13	279 ± 14	276 ± 23	275 ± 21	300 ± 2.4	265 ± 25	278 ± 21	262 ± 17
Mg	49 ± 2.2	50 ± 4.8	45 ± 7.6	50 ± 4.3	49 ± 3.9	52 ± 7.3	53 ± 3.8	54 ± 6.2	54 ± 3.6	52 ± 2.1	54 ± 0.36	51 ± 3.7
Na	$323 \pm 17^{A}_{a}$	$294 \pm 12^{AB}_{a}$	$273 \pm 28^{B}_{a}$	$282 \pm 15^{B}_{a}$	$347 \pm 19^{A}_{ab}$	$239 \pm 22^{B}_{b}$	$240 \pm 22^{B}_{ab}$	$237 \pm 11^{B}_{b}$	$374 \pm 18^{A}_{b}$	$215 \pm 11^{B}_{b}$	$214 \pm 3.3^{B}_{b}$	$203 \pm 20^{B}_{b}$
Liver												
Cu	770 ± 153	$780 \pm 248_{a}$	$729 \pm 157_{a}$	$793 \pm 131_{a}$	854 ± 182^{A}	$1259 \pm 240^{B}_{b}$	$1031 \pm 66^{AB}_{a}$	$965 \pm 89^{AB}_{a}$	717.7 ± 165^{A}	$1589 \pm 174^{B}_{b}$	$1690 \pm 153^{B}_{b}$	$1595 \pm 147^{B}_{b}$
Cd	3.2 ± 0.55^{A}	4.5 ± 1.2^{AB}	$5.5 \pm 0.99^{AB}_{a}$	$8.1 \pm 1.2^{B}_{a}$	4.2 ± 1.7^{A}	4.9 ± 0.50^{AB}	$8.3 \pm 1.4^{B}_{a}$	$14 \pm 3.2^{C}_{b}$	4.8 ± 0.7^{A}	7.9 ± 1.2^{A}	$18 \pm 1.4^{B}_{b}$	$27 \pm 3.7^{C}_{c}$
Ca	7.8 ± 2.6	4.9 ± 0.61	5.0 ± 0.9	4.2 ± 0.28	8.7 ± 4.1^{A}	5.7 ± 2.2^{AB}	5.5 ± 1.9^{AB}	3.9 ± 0.56^{B}	5.3 ± 0.61	3.7 ± 0.83	4.0 ± 0.28	3.7 ± 0.40
K	275 ± 6.7^{A}	$257 \pm 14^{AB}_{a}$	$242 \pm 25^{B}_{a}$	$256 \pm 3.3^{AB}_{a}$	259 ± 15^{A}	$220 \pm 14^{B}_{b}$	$223 \pm 9.5^{B}_{ab}$	$218 \pm 11^{B}_{b}$	283 ± 17^{A}	$190 \pm 11^{B}_{c}$	$206 \pm 7.7^{B}_{b}$	$194 \pm 5.0^{B}_{b}$
Mg	$39 \pm 0.71_{a}$	$39 \pm 1.2_{a}$	$36 \pm 3.4_{a}$	$37 \pm 2.8_{a}$	$34 \pm 2.0_{b}$	$34 \pm 0.72_{b}$	$34 \pm 1.1_{ab}$	$33 \pm 1.5_{b}$	$36 \pm 1.8^{A}_{ab}$	$30 \pm 1.9^{B}_{c}$	$31 \pm 0.40^{B}_{b}$	$30 \pm 1.1^{B}_{b}$
Na	170 ± 19^{A}	$145 \pm 17^{AB}_{a}$	$138 \pm 23^{B}_{a}$	126 ± 7.9^{B}	155 ± 19^{A}	$119 \pm 16^{B}_{ab}$	$133 \pm 19^{AB}_{ab}$	109 ± 8.5^{B}	149 ± 12^{A}	$90 \pm 6.3^{B}_{b}$	$104 \pm 6.7^{B}_{b}$	97 ± 5.4^{B}
Brain												
Cu	106 ± 6.7	110 ± 14	104 ± 15	105 ± 5.3	114 ± 13	102 ± 6.9	97 ± 4.9	99 ± 8.1	104 ± 3.3	99 ± 3.8	103 ± 10	101 ± 11
Cd	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL
Ca	165 ± 180	102 ± 87	27 ± 14	19 ± 4.9	19.7 ± 7.7	186 ± 159	110 ± 84	83 ± 78	121 ± 96	20 ± 5.8	62 ± 64	348 ± 273
K	393 ± 8.6^{A}	$359 \pm 12^{B}_{a}$	$365 \pm 10^{B}_{a}$	359 ± 12^{B}	386 ± 9.8^{A}	$359 \pm 14^{B}_{ab}$	$355 \pm 3.3^{B}_{ab}$	354 ± 9.6^{B}	373 ± 5.6^{A}	$333 \pm 13^{B}_{b}$	$338 \pm 11^{B}_{b}$	339 ± 16^{B}
Mg	32 ± 1.3	31 ± 1.2	31 ± 1.3	31 ± 0.98	30 ± 0.64^{A}	32 ± 1.3^{B}	31 ± 0.34^{AB}	31 ± 0.79^{AB}	29.8 ± 0.94	31 ± 0.83	31 ± 0.59	30 ± 1.0
Na	253 ± 12	$238 \pm 8.0_{a}$	$240 \pm 15_{a}$	$239 \pm 14_{a}$	249 ± 6.5^{A}	$212 \pm 3.3^{B}_{b}$	$214 \pm 7.5^{B}_{b}$	$212 \pm 6.8^{B}_{b}$	249 ± 8.9^{A}	$194 \pm 9.0^{B}_{b}$	$205 \pm 1.1^{B}_{b}$	$201 \pm 7.6^{B}_{b}$
Muscle												
Cu	39 ± 4.3	44 ± 6.4	35 ± 1.8	35 ± 4.5	37 ± 8.7	44 ± 8.1	43 ± 13	48 ± 5.7	45 ± 13	38 ± 6.9	44 ± 2.1	40 ± 4.3
Cd	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL
Ca	41 ± 12	39 ± 15	34 ± 4.6	34 ± 16	36 ± 22	56 ± 25	50 ± 14	56 ± 16	50 ± 20	56 ± 15	61 ± 9.5	55 ± 11
K	383 ± 24	378 ± 37	383 ± 26	367 ± 23	378 ± 74	403 ± 22	389 ± 60	388 ± 24	385 ± 15	370 ± 24	385 ± 21	377 ± 36
Mg	62 ± 2.7	64 ± 2.5	62 ± 1.8	63 ± 2.3	60 ± 12	68 ± 3.5	63 ± 8	65 ± 2.2	63 ± 3.4	65 ± 1.4	67 ± 3.3	65 ± 4.8
Na	87 ± 6.6	$73 \pm 7.7_{a}$	73 ± 9.3	$72 \pm 7.5_{a}$	75 ± 17	$66 \pm 5.1_{ab}$	63 ± 9.4	$65 \pm 0.31_{ab}$	80 ± 7.2^{A}	$49 \pm 5.4^{B}_{b}$	58 ± 9.5^{B}	$50 \pm 1.8^{B}_{b}$
Carcass												
Cu	58 ± 3.0	62 ± 6.0	$52 \pm 7.5_{a}$	57 ± 4.8	60 ± 9.4	67 ± 3.8	$64 \pm 3.2_{ab}$	62 ± 3.5	50.4 ± 2.5^{A}	70 ± 8.0^{B}	$68 \pm 6.1^{B}_{b}$	59 ± 7.1^{AB}
Cd	0.68 ± 0.15	0.96 ± 0.38	$1.0 \pm 0.51_{a}$	$1.6 \pm 0.77_{a}$	0.62 ± 0.22^{A}	1.6 ± 0.18^{AB}	$2.2 \pm 0.74^{BC}_{ab}$	$3.2 \pm 0.30^{C}_{b}$	0.62 ± 0.09^{A}	1.8 ± 0.52^{AB}	$3.1 \pm 0.95^{BC}_{b}$	$4.1 \pm 1.4^{C}_{b}$
Ca	884 ± 93	870 ± 402	697 ± 378	680 ± 309	848 ± 332	970 ± 147	901 ± 331	982 ± 231	1119 ± 103	839 ± 290	941 ± 353	1037 ± 409
K	310 ± 11	310 ± 33	313 ± 20	308 ± 13	339 ± 26	310 ± 17	313 ± 16	313 ± 14	347 ± 11^{A}	293 ± 23^{B}	300 ± 17^{B}	304 ± 15^{B}
Mg	54 ± 4.1	58 ± 12	57 ± 7.5	56 ± 4.7	59 ± 9.8	62 ± 7.0	64 ± 4.5	65 ± 6.8	64 ± 3.4	60 ± 4.0	63 ± 7.6	65 ± 6.5
Na	$229 \pm 11_{a}$	$228 \pm 36_{a}$	220 ± 18	211 ± 4.6	$258 \pm 24^{A}_{ab}$	$198 \pm 13^{B}_{ab}$	202 ± 16^{B}	204 ± 17^{B}	$277 \pm 22^{A}_{b}$	$177 \pm 14^{B}_{b}$	188 ± 16^{B}	181 ± 12^{B}

SI - Table 6: Metal concentrations (nmol/g dw) and electrolyte levels (µmol/g dw) in in different tissues of *Cyprinus carpio* exposed to Cd_{fix}/Cu_{var} mixtures for 1, 3 and 7 days. Mean ± SD, N=5. Lower-case letters in subscript indicate significant differences ($p < 0.05$) of treatments among sampling days, capital letters in superscript indicate significant differences ($p < 0.05$) among treatments within the same sampling day.

	Day 1				Day 3				Day 7			
Gill	**Control**	**Cd_{fix}/Cu_{10}**	**Cd_{fix}/Cu_{25}**	**Cd_{fix}/Cu_{50}**	**Control**	**Cd_{fix}/Cu_{10}**	**Cd_{fix}/Cu_{25}**	**Cd_{fix}/Cu_{50}**	**Control**	**Cd_{fix}/Cu_{10}**	**Cd_{fix}/Cu_{25}**	**Cd_{fix}/Cu_{50}**
Cu	48 ± 3.0^{A}	$118 \pm 13^{B}_{a}$	$104 \pm 15^{B}_{a}$	$148 \pm 8.3^{B}_{a}$	$51 \pm 2{,}9^{A}$	$128 \pm 9.2^{B}_{a}$	$164 \pm 21^{B}_{b}$	$138 \pm 8.3^{B}_{a}$	51 ± 8.1^{A}	$213 \pm 15^{B}_{b}$	$294 \pm 44^{C}_{c}$	$255 \pm 47^{BC}_{b}$
Cd	0.38 ± 0.091^{A}	$27 \pm 3.4^{B}_{a}$	$15 \pm 1.3^{BC}_{a}$	$10 \pm 0.89^{AC}_{a}$	0.32 ± 0.057^{A}	$68 \pm 9.0^{B}_{b}$	$40 \pm 4.0^{C}_{b}$	$18 \pm 2.0^{D}_{ab}$	0.32 ± 0.065^{A}	$80 \pm 15^{B}_{b}$	$51 \pm 8.9^{C}_{b}$	$30 \pm 3.6^{D}_{b}$
Ca	723 ± 102^{AB}	652 ± 49^{AB}	548 ± 126^{B}	779 ± 140^{A}	684 ± 87	684 ± 37	560 ± 76	728 ± 117	753 ± 93^{A}	720 ± 60^{AB}	545 ± 64^{B}	671 ± 86^{AB}
K	272 ± 17	280 ± 3.1	266 ± 29	261 ± 7.2	278 ± 11	259 ± 2.8	276 ± 23	272 ± 5.5	271 ± 34	269 ± 5.3	278 ± 21	271 ± 18
Mg	58 ± 4.0^{A}	55 ± 3.2^{AB}	45 ± 7.6^{B}	62 ± 6.6^{A}	54 ± 4.4	56 ± 2.4	51 ± 3.8	60 ± 1.7	57 ± 7.4	57 ± 2.9	54 ± 0.36	57 ± 3.9
Na	336 ± 14^{A}	281 ± 8.5^{B}	$273 \pm 28^{B}_{a}$	$259 \pm 33^{B}_{a}$	354 ± 13^{A}	259 ± 1.9^{B}	$240 \pm 22^{B}_{ab}$	$250 \pm 13^{B}_{ab}$	354 ± 59^{A}	237 ± 14^{B}	$214 \pm 3.4^{B}_{b}$	$205 \pm 18^{B}_{b}$
Liver												
Cu	870.6 ± 163	1021 ± 94	$729 \pm 157_{a}$	$1074 \pm 256_{a}$	818 ± 137	1094 ± 150	$1031 \pm 66_{a}$	$940 \pm 76_{a}$	874 ± 111^{A}	1397 ± 183^{B}	$1690 \pm 153^{B}_{b}$	$1678 \pm 384^{B}_{b}$
Cd	6.9 ± 0.87^{AB}	$11 \pm 1.6^{A}_{a}$	$5.5 \pm 0.99^{B}_{a}$	8.9 ± 1.9^{AB}	5.3 ± 0.30^{A}	$11.4 \pm 1.8^{B}_{a}$	$8.3 \pm 1.4^{AB}_{a}$	8.2 ± 1.2^{AB}	7.2 ± 1.3^{A}	$26 \pm 6.2^{B}_{b}$	$18 \pm 1.4^{C}_{b}$	9.4 ± 1.8^{A}
Ca	4.8 ± 0.47	7.2 ± 2.8	5.0 ± 0.92	5.7 ± 1.4	7.6 ± 4.4	9.7 ± 5.8	5.5 ± 1.9	6.7 ± 2.7	6.7 ± 2.8	5.2 ± 1.8	4.0 ± 0.28	4.2 ± 0.57
K	265 ± 18^{A}	$286 \pm 31^{A}_{a}$	242 ± 25^{A}	$260 \pm 17^{A}_{a}$	278 ± 1.1^{A}	$229 \pm 12^{AB}_{b}$	223 ± 9.5^{B}	$223 \pm 35^{B}_{ab}$	291 ± 9.0^{A}	$215 \pm 18^{B}_{b}$	206 ± 7.7^{B}	$196 \pm 41^{B}_{b}$
Mg	36 ± 3.1^{A}	$40 \pm 4.4^{AB}_{a}$	36 ± 3.4^{A}	$44 \pm 3.9^{B}_{a}$	37 ± 1.2	$33 \pm 1.4_{b}$	34 ± 1.1	$34 \pm 4.8_{b}$	37 ± 1.8	$33 \pm 2.3_{b}$	32 ± 1.4	$33 \pm 1.1_{b}$
Na	135 ± 18	132 ± 26	138 ± 23	$144 \pm 21_{a}$	152 ± 18	121 ± 18	133 ± 19	$126 \pm 18_{ab}$	154 ± 7.5^{A}	117 ± 13^{AB}	104 ± 6.7^{B}	$102 \pm 24^{B}_{b}$
Brain												
Cu	125 ± 19	115 ± 13	104 ± 15	120 ± 12	119 ± 11^{AB}	127 ± 6.1^{A}	97 ± 4.9^{B}	132 ± 15^{A}	114 ± 8.7	112 ± 8.6	103 ± 10	120 ± 2.6
Cd	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL
Ca	97 ± 98	117 ± 97	27 ± 14	61 ± 80	22 ± 7.9	48 ± 36	110 ± 84	129 ± 125	31 ± 25	112 ± 92	62 ± 64	160 ± 207
K	375 ± 6.3^{A}	356 ± 14^{A}	$365\pm 10^{A}_{a}$	$307 \pm 13^{B}_{a}$	375 ± 11^{A}	371 ± 11^{AB}	$355 \pm 3.3^{AB}_{ab}$	$349 \pm 3.0^{B}_{b}$	376 ± 8.5^{A}	350 ± 15^{B}	$338 \pm 11^{BC}_{b}$	$327 \pm 7.0^{C}_{ab}$
Mg	29 ± 0.34	29 ± 1.2	31 ± 1.3	$28 \pm 1.0_{a}$	29 ± 1.1^{A}	31 ± 1.7^{AB}	31 ± 0.34^{AB}	$34 \pm 4.7^{B}_{b}$	29 ± 0.65	29 ± 1.1	31 ± 0.59	$30 \pm 1.2_{a}$
Na	213 ± 43	216 ± 12	240 ± 15	215 ± 19	242 ± 12	214 ± 15	214 ± 7.6	212 ± 31	239 ± 9.2^{A}	201 ± 5.6^{AB}	205 ± 1.1^{AB}	176 ± 2.9^{B}
Muscle												
Cu	43 ± 6.6	44 ± 3.6	35 ± 1.8	42 ± 5.3	41 ± 9.5	41 ± 5.9	43 ± 13	38 ± 6.3	48 ± 4.9^{A}	41 ± 4.8^{AB}	44 ± 2.1^{AB}	33 ± 4.5^{B}
Cd	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL
Ca	46 ± 7.3	44 ± 20	34 ± 4.6	46 ± 8.9	39 ± 12	52 ± 12	50 ± 14	54 ± 26	42 ± 18	37 ± 4.1	61 ± 9.5	49 ± 11
K	360 ± 19	382 ± 8.3	383 ± 26	388 ± 15	362 ± 48	414 ± 11	389 ± 60	346 ± 47	395 ± 12	404 ± 18	385 ± 21	351 ± 38
Mg	57 ± 6.5	62 ± 2.3	62 ± 1.8	62 ± 3.0	59 ± 6.5	64 ± 1.3	64 ± 8.0	60 ± 8.6	62 ± 1.9	65 ± 1.0	67 ± 3.3	61 ± 6.4
Na	75 ± 11	63 ± 6.8	73 ± 9.3	58 ± 6.7	71 ± 9.3	53 ± 4.4	63 ± 9.4	53 ± 14	73 ± 3.8^{A}	46 ± 3.6^{B}	58 ± 9.5^{AB}	41 ± 7.5^{B}
Carcass												
Cu	57 ± 7.1	60 ± 3.4	$52 \pm 7.5_{a}$	$60 \pm 6.2_{a}$	55 ± 5.6^{A}	62 ± 4.3^{AB}	$64 \pm 3.2^{AB}_{ab}$	$75 \pm 8.6^{B}_{b}$	58 ± 5.6^{A}	70 ± 4.5^{AB}	$68 \pm 6.1^{A}_{b}$	$82 \pm 8.7^{B}_{b}$
Cd	0.88 ± 0.23^{A}	$2.4 \pm 0.16^{B}_{a}$	$1 \pm 0.51^{A}_{a}$	$1.3 \pm 0.23^{A}_{a}$	0.84 ± 0.15^{A}	$3.4 \pm 0.44^{B}_{b}$	$2.2 \pm 0.74^{C}_{b}$	$2.3 \pm 0.22^{C}_{b}$	0.77 ± 0.20^{A}	$5.2 \pm 0.19^{B}_{c}$	$3.1 \pm 0.95^{C}_{b}$	$3.1 \pm 0.50^{C}_{b}$
Ca	1245 ± 308^{AB}	1220 ± 172^{AB}	697 ± 378^{A}	1302 ± 321^{B}	1213 ± 114	1246 ± 257	901 ± 331	1366 ± 142	1084 ± 260	1323 ± 86	941 ± 353	1331 ± 277
K	302 ± 6.5^{AB}	293 ± 5.7^{AB}	313 ± 20^{A}	283 ± 4.1^{B}	301 ± 3.9^{AB}	286 ± 11^{AB}	313 ± 16^{A}	282 ± 4.4^{B}	322 ± 13^{A}	305 ± 10.4^{A}	300 ± 16.9^{A}	268 ± 24^{B}
Mg	72 ± 4.3^{A}	71 ± 5.3^{A}	57 ± 7.5^{B}	75 ± 7.4^{A}	70 ± 1.8	73 ± 5.7	64 ± 4.5	75 ± 4.7	69 ± 6.5	68 ± 3.8	63 ± 7.6	68 ± 9.1
Na	267 ± 22^{A}	224 ± 14^{B}	220 ± 18^{B}	212 ± 5.9^{B}	257 ± 19^{A}	207 ± 6.6^{B}	202 ± 16^{B}	207 ± 13^{B}	264 ± 25^{A}	200 ± 12^{B}	188 ± 16^{B}	180 ± 27^{B}

SI - Table 7: Sodium content, net loss, percentage of loss and loss rate in the gills of common carp exposed to binary mixtures Cu_{fix}/Cd_{var} and Cd_{fix}/Cu_{var} for 1, 3 and 7 days.

Cu_{fix}/Cd_{var} Na Content												
	Day 1				Day 3				Day 7			
Exposure	**Control**	**Cu_{25}/Cd_{10}**	**Cu_{25}/Cd_{25}**	**Cu_{25}/Cd_{50}**	**Control**	**Cu_{25}/Cd_{10}**	**Cu_{25}/Cd_{25}**	**Cu_{25}/Cd_{50}**	**Control**	**Cu_{25}/Cd_{10}**	**Cu_{25}/Cd_{25}**	**Cu_{25}/Cd_{50}**
Total Na content (µmol/g dw)	323 ± 15	294 ± 12	273 ± 28	282 ± 15	347 ± 16	239± 21	240 ± 21	237 ± 10	374 ± 15	215 ± 10	214 ± 3	203 ± 20
Net loss (µmol/g dw)	/	29 ± 12	50 ± 28	41 ± 15	/	107 ± 21	106 ± 21	109 ± 10	/	159 ± 10	160 ± 3	171 ± 20
% loss	/	9 ± 4	15 ± 8	13 ± 4	/	31 ± 6	30 ± 6	31 ± 3	/	42 ± 3	43 ± 1	45 ± 5
Loss rate (µmol g-1 dw h-1)	/	1.2 ± 0.5	2.1 ± 1.2	1.7 ± 0.6	/	1.5 ± 0.3	1.5 ± 0.3	1.5 ± 0.1	/	0.9 ± 0.1	1 ± 0	1 ± 0.1

Cd_{fix}/Cu_{var} Na Content												
	Day 1				Day 3				Day 7			
Exposure	**Control**	**Cd_{25}/Cu_{10}**	**Cd_{25}/Cu_{25}**	**Cd_{25}/Cu_{50}**	**Control**	**Cd_{25}/Cu_{10}**	**Cd_{25}/Cu_{25}**	**Cd_{25}/Cu_{50}**	**Control**	**Cd_{25}/Cu_{10}**	**Cd_{25}/Cu_{25}**	**Cd_{25}/Cu_{50}**
Total Na content (µmol/g dw)	336 ± 12	281 ± 8	273 ± 28	259 ± 32	354 ± 11	259 ± 2	240 ± 22	250 ± 13	354 ± 53	237 ± 14	214 ± 3	205 ± 17
Net loss (µmol/g dw)	/	55 ± 8	63 ± 28	77 ± 32	/	95 ± 2	114 ± 22	103 ± 13	/	117 ± 14	140 ± 3	148 ± 17
% loss	/	16 ± 2	19 ± 8	23 ± 9	/	27 ± 0.5	32 ± 6	29 ± 3	/	33 ± 4	39 ± 1	42 ± 5
Loss rate (µmol g-1 dw h-1)	/	2.3 ± 0.4	2.6 ± 1.2	3.2 ± 1.4	/	1.3 ± 0	1.6 ± 0.3	1.4 ± 0.2	/	0.7 ± 0.1	0.8 ± 0	1 ± 0.1

SI - Table 8: Relative target gene mRNA abundance in gills and liver of *Cyprinus carpio* exposed to Cu_{fix}/Cd_{var} mixtures for 1, 3 and 7 days. Mean ± SD, N=4. Lower-case letters in subscript indicate significant differences ($p < 0.05$) of treatments among sampling days, capital letters in superscript indicate significant differences ($p < 0.05$) among treatments within the same sampling day.

Gill	Day 1				Day 3				Day 7			
	Control	Cu_{fix}/Cd_{10}	Cu_{fix}/Cd_{25}	Cu_{fix}/Cd_{50}	Control	Cu_{fix}/Cd_{10}	Cu_{fix}/Cd_{25}	Cu_{fix}/Cd_{50}	Control	Cu_{fix}/Cd_{10}	Cu_{fix}/Cd_{25}	Cu_{fix}/Cd_{50}
H^+-ATPase	1.0 ± 0.14^{A}	1.9 ± 0.14^{B}	$1.8 \pm 0.38^{B}_{ab}$	1.9 ± 0.32^{B}	1.0 ± 0.16	1.4 ± 0.26	$1.2 \pm 0.12_{a}$	1.6 ± 0.26	1.0 ± 0.15^{A}	1.6 ± 0.38^{B}	$2.1 \pm 0.053^{B}_{b}$	1.7 ± 0.32^{B}
Na^+/K^+-ATPase	1.0 ± 0.17^{A}	$1.6 \pm 0.15^{B}_{a}$	$1.4 \pm 0.17^{AB}_{a}$	$1.5 \pm 0.25^{AB}_{a}$	1.0 ± 0.29	$0.7 \pm 0.062_{b}$	$0.87 \pm 0.14_{b}$	$0.77 \pm 0.086_{b}$	1.0 ± 0.22	$1.2 \pm 0.11_{ab}$	$1.3 \pm 0.25_{ab}$	$1.1 \pm 0.32_{ab}$
CTR1	1.0 ± 0.21^{A}	$2.7 \pm 0.41^{B}_{a}$	$2.3 \pm 0.28^{B}_{a}$	$2.0 \pm 0.37^{B}_{ab}$	1.0 ± 0.16	$1.5 \pm 0.31_{b}$	$1.6 \pm 0.19_{b}$	$1.5 \pm 0.21_{b}$	1.0 ± 0.15^{A}	$2.2 \pm 0.42^{B}_{a}$	$2.3 \pm 0.30^{B}_{a}$	$2.2 \pm 0.14^{B}_{a}$
NHE-2	1.0 ± 0.20^{A}	$0.60 \pm 0.063^{B}_{a}$	$0.62 \pm 0.054^{AB}_{a}$	$0.67 \pm 0.15^{AB}_{a}$	1.0 ± 0.16^{A}	$0.21 \pm 0.043^{B}_{b}$	$0.25 \pm 0.08^{B}_{b}$	$0.24 \pm 0.065^{B}_{b}$	1.1 ± 0.39^{A}	$0.37 \pm 0.097^{B}_{a}$	$0.45 \pm 0.18^{B}_{a}$	$0.36 \pm 0.16^{B}_{ab}$
GR	1.0 ± 0.22^{A}	1.9 ± 0.14^{B}	2.2 ± 0.40^{B}	2.1 ± 0.33^{B}	1.0 ± 0.097^{A}	2.0 ± 0.28^{B}	1.7 ± 0.21^{B}	2.0 ± 0.29^{B}	0.72 ± 0.21^{A}	1.6 ± 0.35^{B}	1.8 ± 0.20^{B}	2.0 ± 0.19^{B}
SOD	1.0 ± 0.19	1.1 ± 0.090	$1.2 \pm 0.12_{a}$	1.2 ± 0.095	1.0 ± 0.078^{A}	1.4 ± 0.16^{B}	$1.1 \pm 0.13^{AB}_{a}$	1.3 ± 0.18^{AB}	1.0 ± 0.14^{A}	1.4 ± 0.15^{B}	$1.6 \pm 0.17^{B}_{b}$	1.3 ± 0.22^{AB}
CAT	1.0 ± 0.085	$0.86 \pm 0.094_{a}$	0.97 ± 0.21	0.97 ± 0.13	1.0 ± 0.10	$1.3 \pm 0.29_{ab}$	1.3 ± 0.18	1.4 ± 0.33	1.0 ± 0.28	$1.4 \pm 0.23_{b}$	1.4 ± 0.15	1.1 ± 0.23
MT	1.0 ± 0.14^{A}	6.2 ± 0.47^{B}	9.5 ± 2.3^{B}	9.4 ± 2.1^{B}	1.0 ± 0.21^{A}	4.0 ± 0.85^{AB}	6.6 ± 0.84^{B}	11 ± 1.3^{C}	1.0 ± 0.28^{A}	4.3 ± 0.30^{B}	6.8 ± 2.6^{BC}	8.1 ± 1.1^{C}
GST	1.0 ± 0.29	1.1 ± 0.34	1.3 ± 0.38	1.2 ± 0.12	1.0 ± 0.20	1.1 ± 0.36	1.1 ± 0.20	1.2 ± 0.25	1.0 ± 0.29	1.1 ± 0.16	1.1 ± 0.12	0.92 ± 0.30
Liver												
GR	1.1 ± 0.42	0.81 ± 0.069	0.98 ± 0.20	0.82 ± 0.17	1.03 ± 0.26	0.90 ± 0.28	1.01 ± 0.16	0.83 ± 0.32	1.1 ± 0.38	1.3 ± 0.12	1.1 ± 0.16	1.3 ± 0.18
SOD	1.0 ± 0.088	0.99 ± 0.10	1.2 ± 0.14	1.0 ± 0.3	1.0 ± 0.13	0.89 ± 0.13	1.14 ± 0.24	1.04 ± 0.085	1.0 ± 0.17	0.85 ± 0.11	1.1 ± 0.13	1.1 ± 0.13
CAT	1.0 ± 0.15	0.75 ± 0.099	$0.80 \pm 0.092_{a}$	$0.80 \pm 0.037_{a}$	1.0 ± 0.16	0.96 ± 0.073	$1.2 \pm 0.18_{b}$	$1.1 \pm 0.22_{ab}$	1.0 ± 0.083^{AB}	0.84 ± 0.18^{A}	$1.0 \pm 0.041^{AB}_{ab}$	$1.18 \pm 0.17^{B}_{b}$
MT	1.0 ± 0.084	$0.63 \pm 0.14_{a}$	$0.90 \pm 0.31_{a}$	$0.60 \pm 0.19_{a}$	1.0 ± 0.20^{A}	$4.5 \pm 1.4^{B}_{b}$	$4.1 \pm 0.62^{B}_{b}$	$4.8 \pm 0.88^{B}_{b}$	1.0 ± 0.27	$1.5 \pm 0.55_{a}$	$1.7 \pm 0.31_{a}$	$2.4 \pm 0.71_{c}$
GST	1.0 ± 0.29	0.82 ± 0.29	1.2 ± 0.22	0.92 ± 0.34	1.1 ± 0.47^{A}	0.34 ± 0.018^{B}	0.43 ± 0.093^{AB}	0.45 ± 0.12^{AB}	1.0 ± 0.16	0.87 ± 0.64	0.79 ± 0.21	0.54 ± 0.13

SI - Table 9: Relative target gene mRNA abundance in gills and liver of *Cyprinus carpio* exposed to Cd_{fix}/Cu_{var} mixtures for 1, 3 and 7 days. Mean ± SD, N=4. Lower-case letters in subscript indicate significant differences ($p < 0.05$) of treatments among sampling days, capital letters in superscript indicate significant differences ($p < 0.05$) among treatments within the same sampling day.

Gill	Day 1				Day 3				Day 7			
	Control	**Cd_{fix}/Cu_{10}**	**Cd_{fix}/Cu_{25}**	**Cd_{fix}/Cu_{50}**	**Control**	**Cd_{fix}/Cu_{10}**	**Cd_{fix}/Cu_{25}**	**Cd_{fix}/Cu_{50}**	**Control**	**Cd_{fix}/Cu_{10}**	**Cd_{fix}/Cu_{25}**	**Cd_{fix}/Cu_{50}**
H^+-ATPase	1.0 ± 0.32^{A}	1.7 ± 0.14^{AB}	$2.4 \pm 0.38^{B}_{ab}$	2.3 ± 0.52^{B}	1.0 ± 0.10^{A}	1.3 ± 0.25^{A}	$1.8 \pm 0.18^{AB}_{a}$	2.5 ± 0.45^{B}	1.0 ± 0.10^{A}	1.9 ± 0.51^{B}	$2.7 \pm 0.47^{B}_{b}$	2.2 ± 0.13^{B}
Na^+/K^+-ATPase	1.0 ± 0.25^{A}	$1.6 \pm 0.26^{B}_{a}$	$1.7 \pm 0.13^{B}_{a}$	1.5 ± 0.21^{B}	1.0 ± 0.032^{A}	$0.86 \pm 0.18^{A}_{b}$	$1.0 \pm 0.19^{A}_{b}$	1.2 ± 0.29^{A}	1.0 ± 0.15^{AB}	$0.86 \pm 0.16^{A}_{b}$	$1.5 \pm 0.14^{B}_{ab}$	1.2 ± 0.23^{AB}
CTR1	1.0 ± 0.27^{A}	2.4 ± 0.96^{B}	$3.7 \pm 0.33^{B}_{ab}$	$2.3 \pm 0.70^{AB}_{a}$	1.0 ± 0.23^{A}	1.5 ± 0.36^{AB}	$2.8 \pm 0.55^{B}_{a}$	$2.5 \pm 0.22^{B}_{a}$	1.0 ± 0.24^{A}	2.7 ± 0.46^{B}	$4.4 \pm 0.58^{C}_{b}$	$4.4 \pm 0.97^{C}_{b}$
NHE-2	1.1 ± 0.41	1.0 ± 0.43	0.58 ± 0.20	0.62 ± 0.22	1.0 ± 0.22^{A}	0.74 ± 0.15^{AB}	0.27 ± 0.064^{B}	0.41 ± 0.12^{B}	1.0 ± 0.072^{A}	0.46 ± 0.24^{AB}	0.45 ± 0.13^{AB}	0.28 ± 0.12^{B}
GR	1.0 ± 0.11^{A}	2.2 ± 0.50^{B}	2.5 ± 0.22^{B}	$2.4 \pm 0.15^{B}_{a}$	1.0 ± 0.19^{A}	1.4 ± 0.15^{AB}	2.3 ± 0.20^{B}	$1.9 \pm 0.17^{AB}_{a}$	1.0 ± 0.17^{A}	2.1 ± 0.42^{B}	2.7 ± 0.60^{B}	$4.5 \pm 0.99^{C}_{b}$
SOD	1.0 ± 0.19	1.4 ± 0.092	1.3 ± 0.064	$0.98 \pm 0.21_{a}$	1.0 ± 0.15	1.3 ± 0.23	1.4 ± 0.036	$1.4 \pm 0.24_{b}$	1.0 ± 0.11^{A}	1.4 ± 0.14^{AB}	1.6 ± 0.28^{B}	$1.6 \pm 0.046^{B}_{b}$
CAT	1.0 ± 0.14^{AB}	1.2 ± 0.13^{A}	0.96 ± 0.20^{AB}	0.65 ± 0.20^{B}	1.0 ± 0.074	1.1 ± 0.063	1.0 ± 0.095	0.96 ± 0.15	1.0 ± 0.13	1.1 ± 0.19	1.1 ± 0.038	0.92 ± 0.15
MT	1.0 ± 0.31^{A}	7.7 ± 2.9^{B}	$11.5 \pm 1.3^{B}_{a}$	8.9 ± 2.0^{B}	1.0 ± 0.15^{A}	6.0 ± 1.5^{B}	$6.5 \pm 1.5^{B}_{b}$	5.5 ± 1.1^{B}	1.0 ± 0.25^{A}	7.4 ± 1.9^{B}	$6.2 \pm 1.9^{B}_{b}$	5.2 ± 1.1^{B}
GST	1.0 ± 0.19	1.3 ± 0.50	0.94 ± 0.19	$1.2 \pm 0.44_{a}$	1.0 ± 0.13	1.0 ± 0.22	0.84 ± 0.18	$0.74 \pm 0.22_{ab}$	1.0 ± 0.13	0.71 ± 0.072	0.71 ± 0.075	$0.46 \pm 0.12_{b}$
Liver												
GR	1.1 ± 0.53	1.2 ± 0.46	0.92 ± 0.078	1.2 ± 0.54	1.0 ± 0.38	0.70 ± 0.12	0.87 ± 0.32	0.82 ± 0.12	1.0 ± 0.097	0.72 ± 0.22	0.75 ± 0.16	0.81 ± 0.26
SOD	1.0 ± 0.12	1.0 ± 0.17	0.97 ± 0.12	0.92 ± 0.23	1.0 ± 0.28	1.0 ± 0.29	1.1 ± 0.26	0.74 ± 0.070	1.0 ± 0.11	0.78 ± 0.21	0.99 ± 0.19	0.80 ± 0.21
CAT	1.0 ± 0.17^{A}	1.0 ± 0.11^{A}	0.88 ± 0.089^{AB}	$0.49 \pm 0.13^{B}_{a}$	1.02 ± 0.24^{A}	0.96 ± 0.21^{A}	1.1 ± 0.31^{A}	$0.48 \pm 0.11^{B}_{a}$	1.0 ± 0.19	0.71 ± 0.093	0.88 ± 0.15	$1.0 \pm 0.22_{b}$
MT	1.0 ± 0.37	1.2 ± 0.36	$0.44 \pm 0.18_{a}$	$0.37 \pm 0.14_{a}$	1.1 ± 0.37^{A}	1.5 ± 0.18^{AB}	$2.5 \pm 0.93^{B}_{b}$	$2.3 \pm 0.80^{AB}_{b}$	1.0 ± 0.21^{A}	1.5 ± 0.51^{A}	$2.1 \pm 0.29^{A}_{b}$	$3.7 \pm 0.91^{B}_{c}$
GST	1.0 ± 0.098^{A}	$1.4 \pm 0.48^{AB}_{a}$	$2.0 \pm 0.50^{B}_{a}$	0.78 ± 0.34^{A}	1.1 ± 0.57^{A}	$0.44 \pm 0.033^{AB}_{b}$	$0.50 \pm 0.19^{AB}_{b}$	0.23 ± 0.039^{B}	1.0 ± 0.18	$0.82 \pm 0.10_{ab}$	$0.92 \pm 0.22_{b}$	0.68 ± 0.22

SI - Table 10: Set of primers (F=forward; R=reverse) designed for common carp using Primer blast (NCBI) or taken from other studies and used for gene expression analysis by quantitative RT-PCR. Reference genes: elongation factor 1α (eEF) and β-actin; target genes: H^+-ATPase, catalase (CAT), superoxide dismutase Cu-Zn (SOD), glutathione reductase (GR), glutathione S-transferase (GST), copper transporter 1 (CTR1), metallothionein (MT), Na^+/H^+-exchanger (NHE-2) and Na^+/K^+-ATPase. Accession numbers for MT and GST were not reported in the original manuscripts.

Gene	Accession number	Primer 5'→ 3'	T_m (°C)	% GC	% efficiency
eEF	Sinha et al. (2012) AF485331.1	F - TGGAGATGCTGCCATTGT R - TGCAGACTTCGTGACCTT	54 54	50 50	92
β-actin	Wu et al. (2014) M24113	F - CGTGATGGACTCTGGTGATG R - TCGGCTGTGGTGGTGAAG	62 58	55 61.1	96
H^+-ATPase	Sinha et al. 2016 JX570880	F - CTATGGGGGTCAACATGGAG R - CCAACACGTGCTTCTCACAC	62 62	55 55	103
CAT	Wu et al. 2014 JF411604	F - CTGGAAGTGGAATCCGTTTG R - CGACCTCAGCGAAATAGTTG	60 60	50 50	98
GR	Wu et al. 2014 JF411607	F - GAGAAGTACGACACCATCCA R - CACACCTATTGAACTGAGATTGAG	60 48.9	50 41.7	106
GST	Casatta et al. 2017	F - GAAACCAGTTGAGCCGTGC R - CGGCTGGAGAAACTTGCTGAT	60 49.2	57.9 52.4	97
SOD	Wu et al. 2014 JF342355	F - TGGCGAAGAAGGCTGTTTGT R - TTCACTGGAGACCCGTCACT	60 62	50 55	93
MT	Reynders et al.	F - CCAAGACTGGAACTTGC R - ACGTTGACCTCCTCAC	52 50	52.9 56.3	93
Na^+/K^+-ATPase	Castaldo et al. 2020 JX570881.1	F - ATGGGTCGTATCGCCACTCT R - CCAGGAAGACAGCAACACCA	62 62	55 55	104
NHE-2	Castaldo et al. 2020 XM_019098528	F - CACACAAGCTTACGACGCAG R - TCCAGTGTGAACGAGTCTCC	59.8 59	55 55	107
CTR1	XM_019104458.1	F - TCATCAACACACCAGGAGGA R - AATAGGAACTCACGGGCGAT	58.3 58.9	50 50	97

Chapter 4.

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

Castaldo, G. ; Nguyễn, T.; Town, R. M.; Bervoets, L.; Blust, R.; and De Boeck, G.

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

Modified version of Aquatic Toxicology (2021); 105875

Abstract

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

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

4.1. Introduction

Trace metals are part of a wide variety of pollutants that have increased in the environment as result of anthropogenic activity (Sevcikova et al. 2011). Moreover, they have a long persistence and can accumulate in the food chain (Eisler 1993, Begum et al. 2005). Even though zinc (Zn) is an essential element, being a key component of structural components and proteins (Watanabe et al. 1997), it can cause problems if present at too high or too low concentrations in the organism. One of the main problems associated with Zn pollution is its ability to lead to disruption of physiological and biochemical mechanisms, one of which is the interference with calcium (Ca) homeostasis (Hogstrand et al. 1995, Bury et al. 2003, Loro et al. 2014). Cadmium (Cd), in contrast to Zn, is a non-essential metal with no-known biological role (Zheng et al. 2016, Danabas et al. 2018). The toxic effects caused by this metal are related with disruption of ionoregulation (McGeer et al. 2011), oxidative stress and immunosuppression (Zhang et al. 2017).

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

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

Moreover, glutathione (GSH) plays a crucial role as a chelating agent for metals (Freedman et al. 1989) and in ROS scavenging (Peña-Llopis et al. 2003). The levels of GSH are ensured by the presence of glutathione reductase (GR), which catalyses the reduction of glutathione disulphide (GSSG) thereby maintaining a constant ratio of

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

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

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

4.2. Material and methods

4.2.1. Experimental animals

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

4.2.2. Experimental set-up

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

4.2.3. Sampling procedure

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

4.2.4. Metal bioaccumulation and electrolytes levels

All the samples collected as described above, were stored in pre-weighed Eppendorf bullet tubes, with the exception of the carcasses, which were collected in pre-weighed 50 mL Falcon tubes. Metal and electrolyte content were determined in five samples at each sampling point. In order to check for the accuracy of the procedure six samples of reference material (SRM-2976, mussel tissue, National Institute of Standards and Technology, Gaithersburg, MD, USA) and six blanks were included in the digestion and analysis procedures. Before starting the digestion process, the samples were dried for 48 h, then cooled down in a desiccator for 2 h. After that the dry weight (dw) was recorded with a precision scale (Sartorius SE2, ultra-microbalance). Briefly, the digestion process (Blust et al. 1988, Reynders et al. 2006a) comprised a pre-digestion of 12 h at room temperature with 69 % concentrated HNO_3, followed by a microwave digestion. After that, H_2O_2 was added to further digest the fat component of the tissue, followed by a final microwave step. Similar to the process, described above, carcasses were also digested using 69 % HNO_3 and H_2O_2, however the digestion process was carried out using a hot block (Environmental Express, Charleston, SC, USA). At the end of the digestion process, all the samples were diluted to reach a final acid concentration between 1 - 3% with ultrapure Milli-Q (MQ). Metal content and electrolyte levels were determined respectively with a 7700x ICP-MS (Agilent Technologies, Santa Clara, CA, USA) and an iCAP 6300 Duo (Thermo Scientific, Waltham, MA, USA). Results obtained with ICP-MS and iCAP refer to the total element content (e.g. total Cu, Na). Therefore, ionic charges were only added when relevant for the discussion.

4.2.5. Gene expression

Aliquots of gill and liver samples, collected as described above (~30 - 50 mg), were used for gene expression analysis. Total RNA was extracted according to the manufacturer protocol using Trizol (Invitrogen, Merelbeke, Belgium). In order to

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

4.2.6. Statistical analysis

All data are presented as mean values ± standard deviation (SD). Before any statistical analysis, all data were checked for normality by the Shapiro-Wilk test. If the data were not normally distributed, they were log transformed. Two-way analyses of variance (ANOVA) were performed on the obtained data, followed by Tukey test. For metal concentration values below the minimum quantification limit (Cd: 0.00089 µM or 0.1 µg/L; Zn: 0.015 µM or 1 µg/L) half of the respective quantification limit values were utilized for the statistical analysis (Custer et al. 2000). In case more than 50% of the

observations were BMQL, no statistical tests were conducted. The level of significance for statistical analyses were considered at $p<0.05$. All statistical tests were performed with GraphPad Prism version 8.02 for Windows (GraphPad Software, La Jolla California USA). Data presented in the supplementary information were also analysed using the same software.

4.3. Results

4.3.1. Metal bioaccumulation

4.3.1.1. Zn bioaccumulation

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

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

No significant Zn accumulation was observed in the liver (Fig. 1.1.B and 1.2.B), or in the remaining tissues (see SI- table 7 and 8).

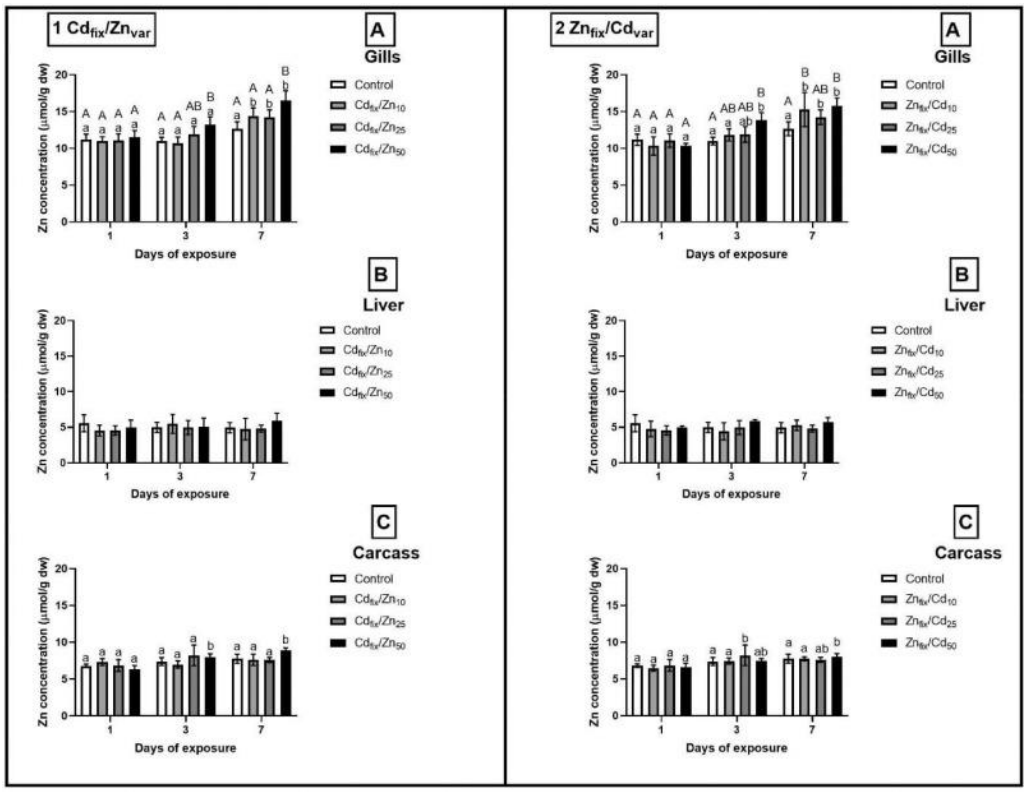


Figure 1: Zn concentration (µmol/g dw) in gills (A), liver (B) and carcass (C) of *Cyprinus carpio* exposed to Cd_{fix}/Zn_{var} (1) or Zn_{fix}/Cd_{var} (2) mixture sampled on day 1, 3 and 7 (mean ± SD, n=5).). Letters were only added when statistical differences occurred. Lower-case letters denote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

4.3.1.2. Cd bioaccumulation

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

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

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

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

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

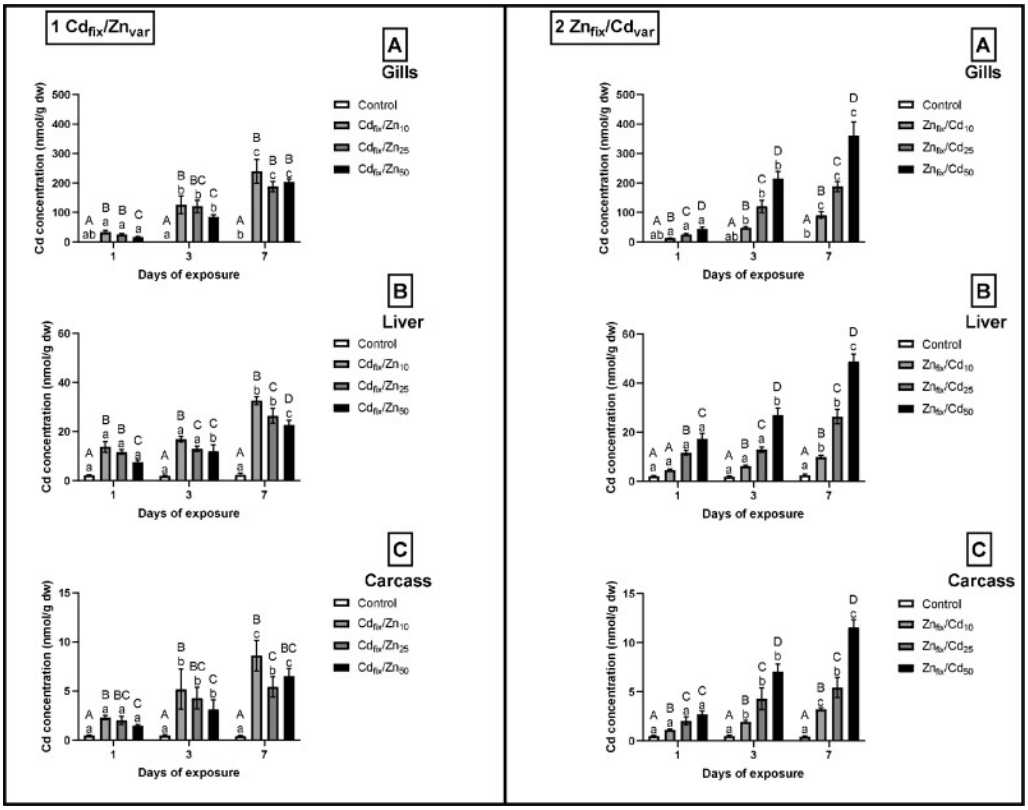


Figure 2: Cd concentration (nmol/g dw) in gills (A), liver (B) and carcass (C) of *Cyprinus carpio* exposed to Cd_{fix}/Zn_{var} (1) or Zn_{fix}/Cd_{var} (2) mixture sampled on day 1, 3 and 7 (mean ± SD, n=5). Letters were only added when statistical differences occurred. Lower-case letters denote significant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

4.3.2. Gene expression

4.3.3. Metallothionein

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

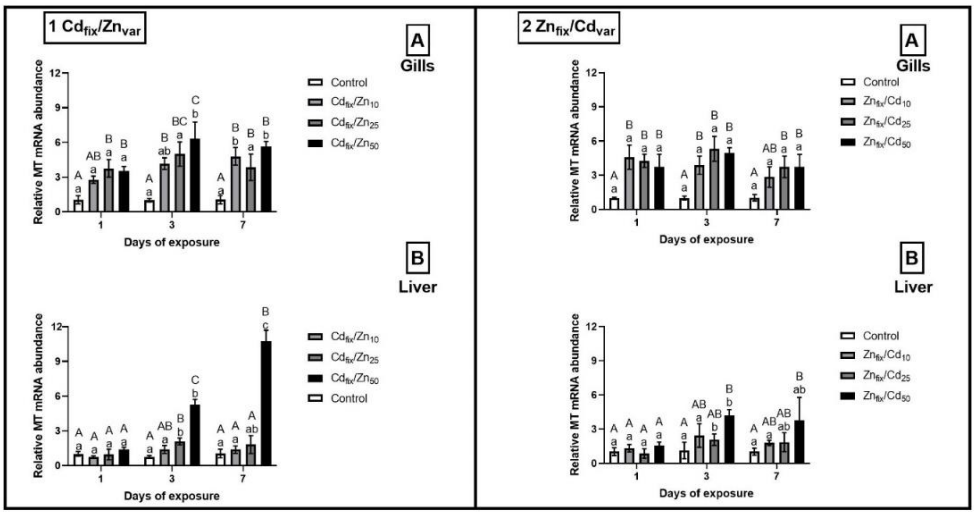


Figure 3: Relative metallothionein (MT) mRNA abundance in gills (A) and liver (B) of *Cyprinus carpio* exposed to Cd_{fix}/Zn_{var} (1) or Zn_{fix}/Cd_{var} (2) mixture sampled on day 1, 3 and 7 (mean ± SD, n=4). Letters were only added when statistical differences occurred. Lower-case letters denote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

4.3.4. Antioxidant enzymes

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

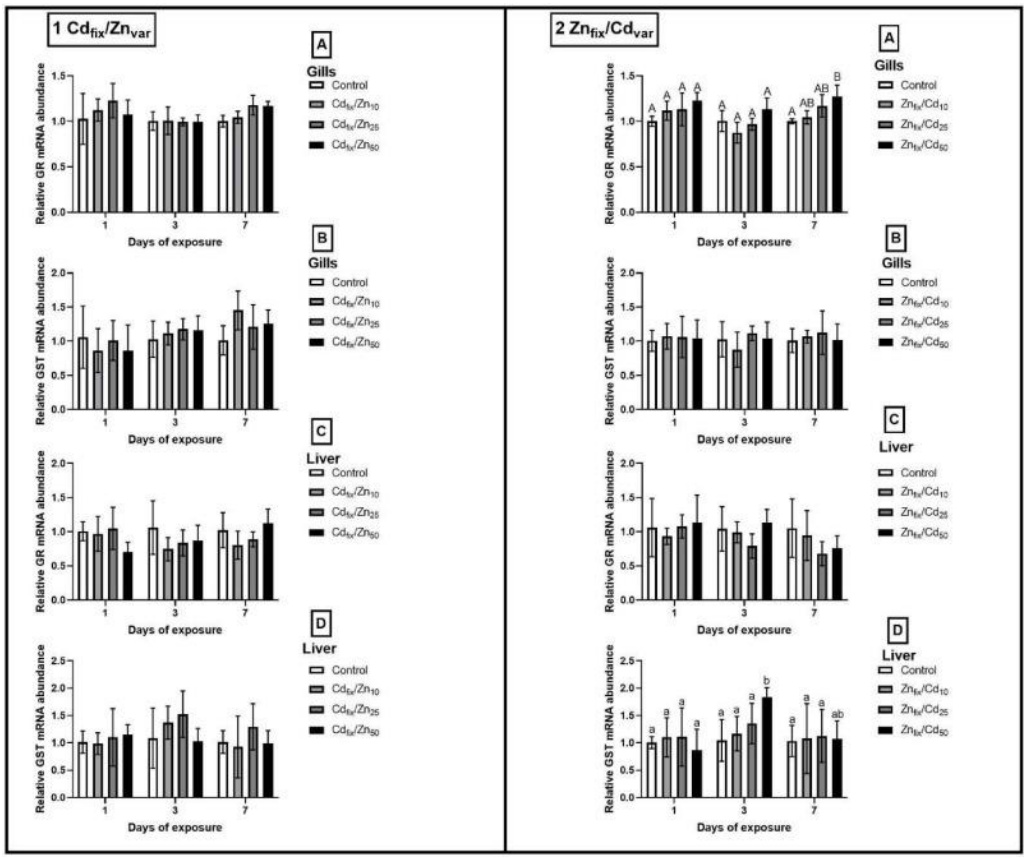


Figure 4: Relative glutathione reductase (GR) and glutathione-S-transferase (GST) mRNA abundance in both gills (A and B, respectively) and liver (C and D, respectively) of *Cyprinus carpio* exposed to Cd_{fix}/Zn_{var} (1) or Zn_{fix}/Cd_{var} (2) mixture sampled on day 1, 3 and 7 (mean ± SD, n=4). Letters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

4.3.5. Indicator of apoptosis

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

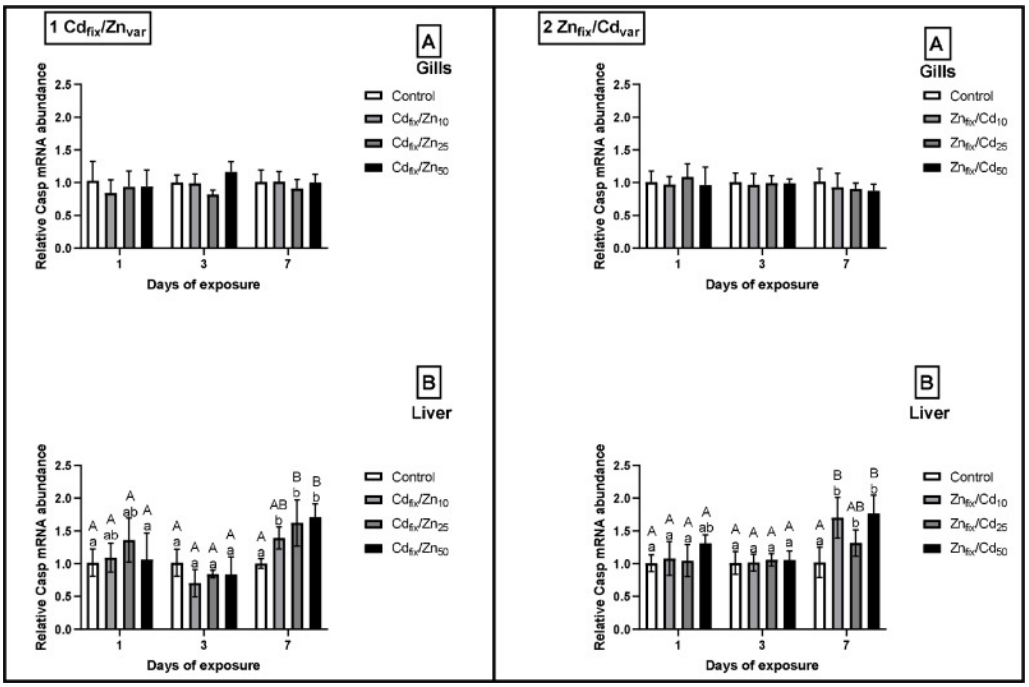


Figure 5: Relative caspase 9 (Casp) mRNA abundance in gills (A) and liver (B) of *Cyprinus carpio* exposed to Cd_{fix}/Zn_{var} (1) or Zn_{fix}/Cd_{var} (2) mixture sampled on day 1, 3 and 7 (mean ± SD, n=4). Letters were only added when statistical differences occurred. Lower-case letters denote significant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

4.3.6. Tissue electrolyte levels

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

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

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

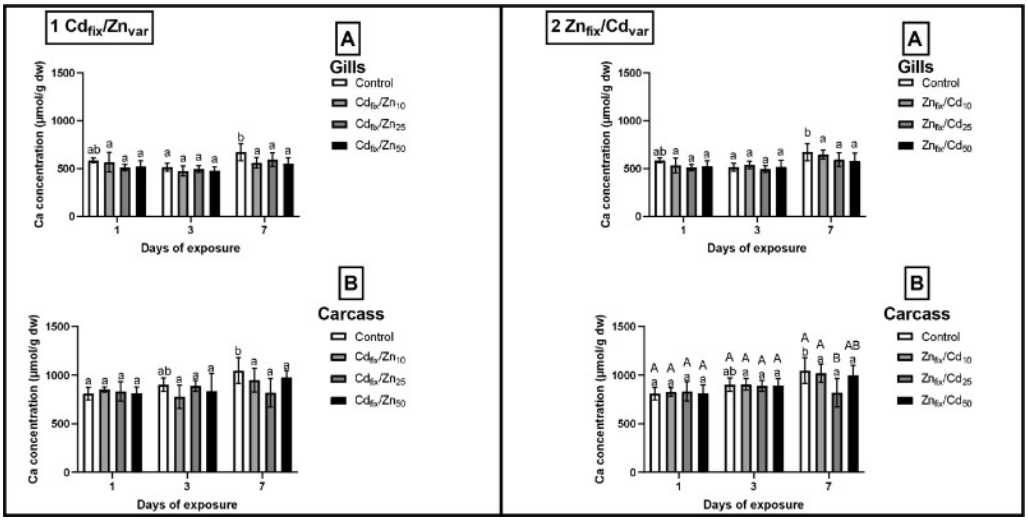


Figure 6: Ca concentration (µmol/g dw) in gills (A) and carcass (B) of *Cyprinus carpio* exposed to Cd_{fix}/Zn_{var} (1) or Zn_{fix}/Cd_{var} (2) mixture sampled on day 1, 3 and 7 (mean ± SD, n=5). Letters were only added when statistical differences occurred. Lower-case letters denote significant differences ($p<0.05$) of treatments between sampling days, capital letters indicate significant differences ($p<0.05$) among treatments within the same sampling day.

4.4. Discussion

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

4.4.1. Metal bioaccumulation

4.4.1.1. Zinc and cadmium bioaccumulation in the gills

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

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

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

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

Therefore, comparing both the results obtained in the single exposure scenario and in the binary mixture, one can assume that in common carp, both metals interfere negligibly to moderately with each other's accumulation. Furthermore, the results obtained at the end of the experiment, at higher waterborne Cd levels, might possibly indicate gill damages at these Cd levels. Nevertheless, more detailed studies using isotopic forms of the metals to differentiate newly accumulated from background metals, are needed to validate these thoughts.

Finally, the difference observed in the mixtures between Zn and Cd accumulation can be linked with the higher affinity that Cd has for gill binding sites as compared to Zn (Playle et al. 1993, Playle 2004). Specifically, Cd binds to the gills approximately 1000

times stronger than Zn under equal exposure conditions (Playle, 2004). Moreover considering that both Zn and Cd have high affinity for cysteine protein in the order Cd > Zn (Saibu et al. 2018), it is reasonable to assume that Cd displaced the Zn bound to these proteins, which was subsequently flushed away.

4.4.1.2. Metals bioaccumulation in the remaining tissues

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

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

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

4.4.2. Defensive mechanisms and indicators of apoptosis

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

The differences in MT induction observed between the two tissues, might be linked with the MT background levels, which are higher in the liver compared to the gills (Hashemi et al. 2008b), and thus might be sufficient to cope with the accumulated metals. Nonetheless, as hypothesized by several authors, the involvement of cytoplasmatic foci, such as the stress granules (Ferro et al. 2015, Chatzidimitriou et al. 2020, Ferro et al. 2020), in which the mRNA is stored for future translation (Lavut and Raveh 2012) can not be excluded. Furthermore as observed in juvenile rainbow trout, even though Cd accumulation on molar basis was not tracked quantitatively by the MT induction, the levels of this protein present in the liver and in the kidney were adequate to complex all the accumulated Cd, whereas this was not the case in the gills (Hollis et al. 2001). This increase in MT gene expression can be thought of as a “state

of readiness", considering that metals are transferred to storage and excretion organs such as liver (Cinier et al. 1999, Arini et al. 2015). In fact as demonstrated by Arini et al. (2015), MTs synthesized in response to a metal exposure can be maintained for several weeks at cellular level. This would clearly represent an advantage in case of persistent metal contamination and higher levels of accumulated metals. This thought appears to be in line with the presence of stress granules, which by stabilizing the mRNA contained in them will allow a faster response to stress. Some recent studies on fish focused on the gene expression of stress granule nucleation proteins seems to confirm this hypothesis (Nicorelli et al. 2018).

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

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

4.4.3. Electrolyte levels

Electrolytes are important for physiological and metabolic processes (Sathya et al. 2012). In the present study, no substantial differences were observed in electrolyte content. For instance, Na and K levels in the present study were not impacted by the metal mixture. Similarly, even though both Cd and Zn are known to compete with Ca^{2+} at the uptake site and inhibiting the Ca^{2+}-ATPase (Hogstrand 2011, McGeer et al. 2011),

no gill Ca loss occurred. Several studies reported a Ca loss in freshwater fish exposed to Cd and Zn, such as rainbow trout and killifish (*Fundulus heteroclitus*) (McGeer et al. 2000b, Loro et al. 2014). Nonetheless, in the carcasses and the muscle some Ca loss was observed. However, this apparent loss appears to be more related with biological variation rather than with the metal exposure. Similarly, also the few differences observed for Mg (e.g. gills and carcasses) seems more due to internal variation rather than the metal exposure. The lack of effects of the metals on Mg content, seems to be in line with Reynders et al. (2006a), who found no changes in plasma Mg content in common carp simultaneously exposed to Cd via water (≃ 0.08, 0.93 and 4 μM) and via food (≃ 0.08, 1.08 and 1.26 μM).

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

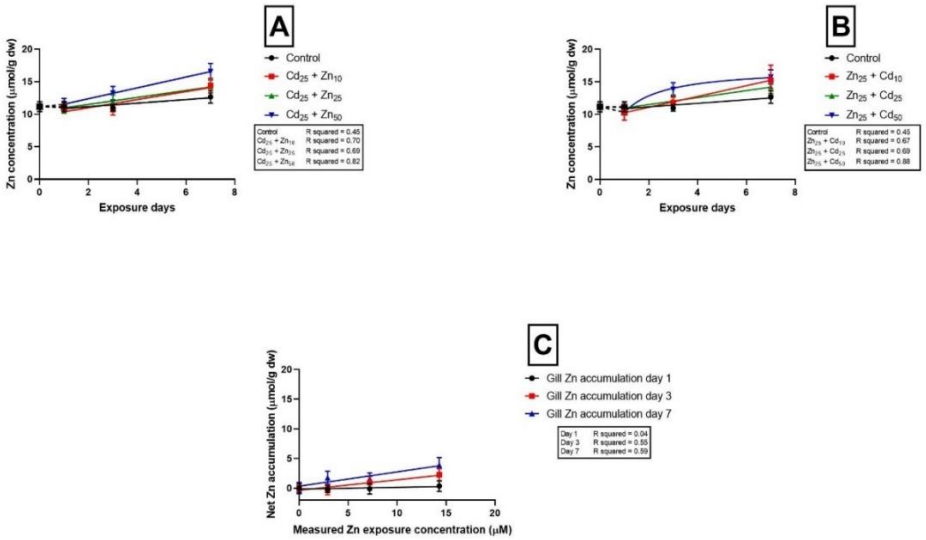
4.5. Conclusions

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

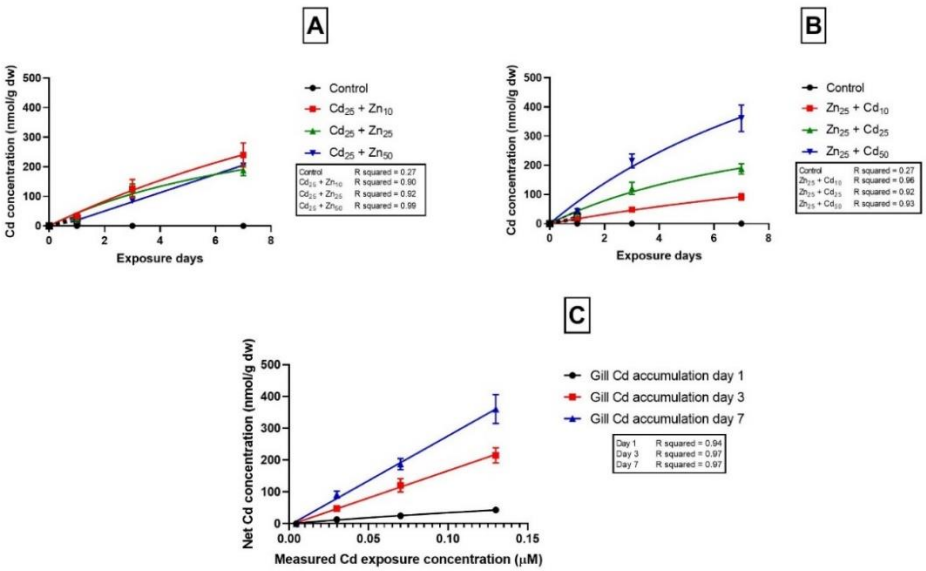
Acknowledgements

We thank Steven Joosen and Karin Van den Bergh and for their technical assistance and the reviewers for their constructive comments.

4.6. Supplementary information



SI-Fig. 1: Zn gills concentration over time in both the Cd_{fix}/Zn_{var} (A) and Zn_{fix}/Cd_{var} (B) exposures, dotted lines indicate assumed accumulation rates during the first day, extrapolating starting values from control values at day 1; (C) net Zn accumulation as function of the concentration in the Cd_{fix}/Zn_{var} exposure. The Michaelis-Menten (Y = Vmax*X/(Km + X)) curves (▼ symbols in the graph B) and lines (Y= YIntercept + X*Slope) were fitted using measured concentration of the variable metal of each experimental series.



SI-Fig. 2: Cd gills concentration over time in both the Cd_{fix}/Zn_{var} (A) and Zn_{fix}/Cd_{var} (B) exposures, dotted lines indicate assumed accumulation rates during the first day, extrapolating starting values from control values at day 1; (C) net Cd accumulation as function of the concentration in the Zn_{fix}/Cd_{var} exposure. The Michaelis-Menten (Y = Vmax*X/(Km + X)) curves and lines (Y= YIntercept + X*Slope) (■ and ▼ symbols in the graphs A and C) were fitted using measured concentration of the variable metal of each experimental series.

SI-Table 1: Total waterborne metal concentration (mean ± SD) measured during the experiment (BMQL = Below Method Quantification Limit).

	Nominal concentration	**Measured concentration**
Control	0 µM Cd 0 µM Zn	BMQL Cd BMQL Zn
Treatment Cd_{fix}/Zn_{10}	25% LC_{50} Cd (0.05 µM) 10% LC_{50} Zn (3 µM)	0.063 ± 0.006 µM Cd 2.9 ± 0.4 µM Zn
Treatment Cd_{fix}/Zn_{25}	25% LC_{50} Cd (0.05 µM) 25% LC_{50} Zn (7.5 µM)	0.066 ± 0.008 µM Cd 7.3 ± 0.9 µM Zn
Treatment Cd_{fix}/Zn_{50}	25% LC_{50} Cd (0.05 µM) 50% LC_{50} Zn (15 µM)	0.066 ± 0.008 µM Cd 14.5 ± 2.3 µM Zn
Treatment Zn_{fix}/Cd_{10}	25% LC_{50} Zn (7.5 µM) 10% LC_{50} Cd (0.02 µM)	7.5 ± 0.6 µM Zn 0.026 ± 0.005 µM Cd
Treatment Zn_{fix}/Cd_{50}	25% LC_{50} Zn (7.5 µM) 50% LC_{50} Cd (0.10 µM)	7.1 ± 1 µM Zn 0.126 ± 0.015 µM Cd

SI-Table 2: Chemical speciation in the mixture of the Cd_{fix}/Zn_{var} exposure, calculated using measured concentrations with the equilibrium speciation code VMinteq.

Component	Concentration (mol/l)	% of total concentration	Species name	Concentration (mol/l)	% of total concentration	Species name
Cd_{25}/Zn_{10}	5.2939E-08	84.030	Cd^{2+}	2.1002E-06	72.422	Zn^{2+}
		0.318	$CdOH^{+}$		0.016	$Zn(CO_3)_2^{2-}$
		0.364	$CdCl^{+}$		3.429	$ZnOH^{+}$
		8.322	$CdSO_4$ (aq)		5.059	$Zn(OH)_2$ (aq)
		0.094	$Cd(SO_4)_2^{2-}$		6.810	$ZnSO_4$ (aq)
		1.828	$CdHCO_3^{+}$		0.049	$Zn(SO_4)_2^{2-}$
		5.026	$CdCO_3$ (aq)		10.633	$ZnCO_3$ (aq)
		0.016	$Cd(CO_3)_2^{2-}$		1.572	$ZnHCO_3^{+}$
Cd_{25}/Zn_{25}	5.5466E-08	84.039	Cd^{2+}	5.2876E-06	72.433	Zn^{2+}
		0.318	$CdOH^{+}$		0.016	$Zn(CO_3)_2^{2-}$
		0.364	$CdCl^{+}$		3.429	$ZnOH^{+}$
		8.317	$CdSO_4$ (aq)		5.059	$Zn(OH)_2$ (aq)
		0.094	$Cd(SO_4)_2^{2-}$		6.806	$ZnSO_4$ (aq)
		1.827	$CdHCO_3^{+}$		0.049	$Zn(SO_4)_2^{2-}$
		5.023	$CdCO_3$ (aq)		10.626	$ZnCO_3$ (aq)
		0.016	$Cd(CO_3)_2^{2-}$		1.571	$ZnHCO_3^{+}$
Cd_{25}/Zn_{50}	5.5475E-08	84.054	Cd^{2+}	1.0505E-05	72.450	Zn^{2+}
		0.318	$CdOH^{+}$		0.016	$Zn(CO_3)_2^{2-}$
		0.364	$CdCl^{+}$		3.429	$ZnOH^{+}$
		8.309	$CdSO_4$ (aq)		5.059	$Zn(OH)_2$ (aq)
		0.093	$Cd(SO_4)_2^{2-}$		6.800	$ZnSO_4$ (aq)
		1.825	$CdHCO_3^{+}$		0.049	$Zn(SO_4)_2^{2-}$
		5.018	$CdCO_3$ (aq)		10.616	$ZnCO_3$ (aq)
		0.016	$Cd(CO_3)_2^{2-}$		1.570	$ZnHCO_3^{+}$

SI-Table 3: Chemical speciation in the mixture of the Zn_{fix}/Cd_{var} exposure, calculated using measured concentrations with the equilibrium speciation code VMinteq.

Component	Concentration (mol/l)	% of total concentration	Species name	Concentration (mol/l)	% of total concentration	Species name
Zn_{25}/Cd_{10}	5.4325E-06	72.433	Zn^{2+}	2.1850E-08	84.040	Cd^{2+}
		0.016	$Zn(CO_3)_2^{2-}$		0.318	$CdOH^+$
		3.429	$ZnOH^+$		0.364	$CdCl^+$
		5.059	$Zn(OH)_2$ (aq)		8.317	$CdSO_4$ (aq)
		6.806	$ZnSO_4$ (aq)		0.094	$Cd(SO_4)_2^{2-}$
		0.049	$Zn(SO_4)_2^{2-}$		1.827	$CdHCO_3^+$
		10.626	$ZnCO_3$ (aq)		5.023	$CdCO_3$ (aq)
		1.571	$ZnHCO_3^+$		0.016	$Cd(CO_3)_2^{2-}$
Zn_{25}/Cd_{25}	5.2876E-06	72.433	Zn^{2+}	5.5466E-08	84.039	Cd^{2+}
		0.016	$Zn(CO_3)_2^{2-}$		0.318	$CdOH^+$
		3.429	$ZnOH^+$		0.364	$CdCl^+$
		5.059	$Zn(OH)_2$ (aq)		8.317	$CdSO_4$ (aq)
		6.806	$ZnSO_4$ (aq)		0.094	$Cd(SO_4)_2^{2-}$
		0.049	$Zn(SO_4)_2^{2-}$		1.827	$CdHCO_3^+$
		10.626	$ZnCO_3$ (aq)		5.023	$CdCO_3$ (aq)
		1.571	$ZnHCO_3^+$		0.016	$Cd(CO_3)_2^{2-}$
Zn_{25}/Cd_{50}	5.1427E-06	72.432	Zn^{2+}	1.0589E-07	84.039	Cd^{2+}
		0.016	$Zn(CO_3)_2^{2-}$		0.318	$CdOH^+$
		3.429	$ZnOH^+$		0.364	$CdCl^+$
		5.059	$Zn(OH)_2$ (aq)		8.317	$CdSO_4$ (aq)
		6.806	$ZnSO_4$ (aq)		0.094	$Cd(SO_4)_2^{2-}$
		0.049	$Zn(SO_4)_2^{2-}$		1.827	$CdHCO_3^+$
		10.627	$ZnCO_3$ (aq)		5.023	$CdCO_3$ (aq)
		1.571	$ZnHCO_3^+$		0.016	$Cd(CO_3)_2^{2-}$

SI-Table 4: Primer sequences (F= forward; R= reverse), Tm°C and efficiency of target and housekeeping genes.

Gene	Accession number	Primer 5' → 3'	Annealing temperature (°C)	Efficiency %
EF1α	Sinha et al. (2012) AF485331.1	F – TGGAGATGCTGCCATTGT	58	92
		R – TGCAGACTTCGTGACCTT		
β-actin	Wu et al. (2014) M24113	F – CGTGATGGACTCTGGTGATG	62	96
		R – TCGGCTGTGGTGGTGAAG		
CAT	Wu et al. (2014) JF411604	F – CTGGAAGTGGAATCCGTTTG	54	103
		R – CGACCTCAGCGAAATAGTTG		
MT	Reynders et al. (2006b)	F – CCAAGACTGGAACTTGC	60	93
		R – ACGTTGACCTCCTCAC		
SOD	Wu et al. (2014) JF342355	F – TGGCGAAGAAGGCTGTTTGT	60	93
		R – TTCACTGGAGACCCGTCACT		
CASP	Pillet et al. (2019) KC676314.1	F – TTGAGGAGAATGCTGCCACG	61	94
		R – TCCCACTGCAGCAAAAAGTG		
GST	DQ411310.1	F – GCTTTCCCAAAATCCAGGCG	60	97.7
		R – TGGCTCAACACCTCCTTCAC		
GR	Wu et al. (2014) JF411607	F – GAGAAGTACGACACCATCCA	60	106
		R – CACACCTATTGAACTGAGATTGAG		
GPx	GQ376155.1	F – TCTCTCAAAGGTAAAGTGGTGCT	60	106.7
		R – GCTCGTTCATCTGGGTGTAATC		

SI-Table 5: Metal content, metal net accumulation, percentage of increase and accumulation rate in the gills of common carp exposed to the binary mixture Cd_{fix}/Zn_{var} for 1, 3 and 7 days.

Cd_{fix}/Zn_{var} Zn Content												
	Day 1				Day 3				Day 7			
Exposure	**Control**	**Cd_{25}/Zn_{10}**	**Cd_{25}/Zn_{25}**	**Cd_{25}/Zn_{50}**	**Control**	**Cd_{25}/Zn_{10}**	**Cd_{25}/Zn_{25}**	**Cd_{25}/Zn_{50}**	**Control**	**Cd_{25}/Zn_{10}**	**Cd_{25}/Zn_{25}**	**Cd_{25}/Zn_{50}**
Total metal content (µmol/g dw)	11.16 ± 0.76	10.99 ± 0.59	11.05 ± 0.91	11.52 ± 0.91	10.99 ± 0.50	10.71 ± 0.83	11.90 ± 1.07	13.24 ± 1.05	12.66 ± 0.95	14.41 ± 1.10	14.23 ± 1.00	16.54 ± 1.26
Net accumulation (µmol /g dw)	/	-0.18 ± 0.59	-0.11 ± 0.91	0.36 ± 0.91		-0.28 ± 0.83	0.91 ± 1.07	2.25 ± 1.05		1.75 ± 1.10	1.57 ± 1	3.88 ± 1.27
% increase	/	-1.57 ± 5.32	-0.97 ± 8.16	3.26 ± 8.14	/	-2.57 ± 7.56	8.24 ± 9.71	20.43 ± 9.51	/	13.79 ± 8.66	12.37 ± 7.90	30.62 ± 9.99
Accumulation rate (µmol g^{-1} dw h^{-1})	/	-0.007 ± 0.025	-0.005 ± 0.038	0.015 ± 0.038	/	-0.004 ± 0.012	0.013 ± 0.015	0.031 ± 0.015	/	0.010 ± 0.007	0.022 ± 0.014	0.023 ± 0.008
Cd_{fix}/Zn_{var} Cd Content												
	Day 1				**Day 3**				**Day 7**			
Exposure	Control	Cd_{25}/Zn_{10}	Cd_{25}/Zn_{25}	Cd_{25}/Zn_{50}	Control	Cd_{25}/Zn_{10}	Cd_{25}/Zn_{25}	Cd_{25}/Zn_{50}	Control	Cd_{25}/Zn_{10}	Cd_{25}/Zn_{25}	Cd_{25}/Zn_{50}
Total metal content (nmol /g dw)	0.27 ± 0.04	33.57 ± 5.63	25.10 ± 3.63	16.76 ± 1.85	0.20 ± 0.04	126.09 ± 30.85	121.00 ± 21.05	85. 20 ± 7.99	0.34 ± 0.07	239.74 ± 40.41	187.66 ± 17.61	204.25 ± 9.33
Net accumulation (nmol /g dw)	/	33.30 ± 5.63	24.83 ± 3.63	16.50 ± 1.85	/	125.89 ± 30.85	120. 81 ± 21.04	85 ± 7.99	/	239.40 ± 40.41	187.31 ± 17.61	203.90 ± 9.33
% increase	/	12513 ± 2114	9330 ± 1364	6196 ± 694	/	64375 ± 15774	61773 ± 10763	43463 ± 4083	/	70045 ± 11824	54805 ± 5153	59660 ± 2730
Accumulation rate (nmol g^{-1} dw h^{-1})	/	1.39 ± 0.24	1.04 ± 0.15	0.69 ± 0.07	/	1.75 ± 0.43	1.68 ± 0.29	1.18 ± 0.11	/	1.43 ± 0.24	1.12 ± 0.11	1.21 ± 0.05

SI-Table 6: Metal content, metal net accumulation, percentage of increase and accumulation rate in the gills of common carp exposed to the binary mixture Zn_{fix}/Cd_{var} for 1, 3 and 7 days.

Zn_{fix}/Cd_{var} Zn Content												
	Day 1				Day 3				Day 7			
Exposure	Control	Zn_{25}/Cd_{10}	Zn_{25}/Cd_{25}	Zn_{25}/Cd_{50}	Control	Zn_{25}/Cd_{10}	Zn_{25}/Cd_{25}	Zn_{25}/Cd_{50}	Control	Zn_{25}/Cd_{10}	Zn_{25}/Cd_{25}	Zn_{25}/Cd_{50}
Total metal content (µmol/g dw)	11.16 ± 0.76	10.32 ± 1.24	11.05 ± 0.91	10.32 ± 0.36	10.99 ± 0.50	11.82 ± 0.84	11.90 ± 1.07	13.84 ± 1.02	12.66 ± 0.95	15.26 ± 2.32	14.23 ± 1.00	15.75 ± 1.10
Net accumulation (µmol /g dw)	/	-0.84 ± 1.24	-0.11 ± 0.91	-0.84 ± 0.36	/	0.83 ± 0.84	0.91 ± 1.07	2.85 ± 1.02	/	2.60 ± 2.32	1.57 ± 1.00	3.09 ± 1.10
% increase	/	-7.56 ± 11.08	-0.97 ± 8.16	-7.55 ± 3.26	/	7.53 ± 7.65	8.24 ± 9.7	25.97 ± 9.24	/	20.57 ± 18.33	12.37 ± 7.90	24.41 ± 9.72
Accumulation rate (µmol g^{-1} dw h^{-1})	/	-0.035 ± 0.052	-0.005 ± 0.038	-0.035 ± 0.015	/	0.012 ± 0.012	0.013 ± 0.015	0.040 ± 0.014	/	0.015 ± 0.014	0.009 ± 0.006	0.018 ± 0.007
Zn_{fix}/Cd_{var} Cd Content												
	Day 1				Day 3				Day 7			
Exposure	Control	Zn_{25}/Cd_{10}	Zn_{25}/Cd_{25}	Zn_{25}/Cd_{50}	Control	Zn_{25}/Cd_{10}	Zn_{25}/Cd_{25}	Zn_{25}/Cd_{50}	Control	Zn_{25}/Cd_{10}	Zn_{25}/Cd_{25}	Zn_{25}/Cd_{50}
Total metal content (nmol/g dw)	0.27 ± 0.04	13.31 ± 0.64	25.10 ± 3.63	43.30 ± 7.36	0.20 ± 0.04	48.06 ± 3.50	121.00 ± 21.05	215.10 ± 24.02	0.34 ± 0.07	91.91 ± 10.99	187.66± 17.61	360.96 ± 45.49
Net accumulation (nmol/g dw)	/	13.04 ± 0.64	24.84 ± 3.63	43.03 ± 7.36	/	47.87 ± 3.50	120.81 ± 21.05	214.91 ± 24.02	/	91.57 ± 10.99	187.31 ± 17.61	360.62 ± 45.49
% increase	/	4898 ± 239	9330 ± 1364	16165 ± 2766	/	24477 ± 1789	61773 ± 10763	109888 ± 12282	/	26791 ± 3215	54805 ± 5153	105512 ± 13310
Accumulation rate (nmol g^{-1} dw h^{-1})	/	0.54 ± 0.03	1.03 ± 0.15	1.79 ± 0.31	/	0.66 ± 0.05	1.68 ± 0.29	2.98 ± 0.33	/	0.55 ± 0.6	1.11 ± 0.11	2.15 ± 0.27

SI-Table 7*:* Zinc (µmol/g dw), Cd (nmol/g dw) and electrolyte levels (µmol/g dw) in gills, liver, brain, muscle and carcass of *Cyprinus carpio* exposed to Cd_{fix}/Zn_{var} mixtures for 1, 3 and 7 days. Mean ± SD, N=5. Lower-case letters indicate significant differences ($p < 0.05$) of treatments among sampling days, capital letters indicate significant differences ($p < 0.05$) among treatments within the same sampling day.

	Day 1				Day 3				Day 7			
Gill	Control	Cd_{fix}/Zn_{10}	Cd_{fix}/Zn_{25}	Cd_{fix}/Zn_{50}	Control	Cd_{fix}/Zn_{10}	Cd_{fix}/Zn_{25}	Cd_{fix}/Zn_{50}	Control	Cd_{fix}/Zn_{10}	Cd_{fix}/Zn_{25}	Cd_{fix}/Zn_{50}
Zn	11.16 ± 0.77^{Aa}	10.99 ± 0.59^{Aa}	11.05 ± 0.91^{Aa}	11.52 ± 0.91^{Aa}	10.99 ± 0.5^{Aa}	10.71 ± 0.83^{Aa}	11.9 ± 1.07^{ABa}	13.24 ± 1.05^{Ba}	12.66 ± 0.95^{Aa}	14.41 ± 1.1^{Ab}	14.23 ± 1^{Ab}	16.54 ± 1.27^{Bb}
Cd	0.27 ± 0.04^{Aab}	33.57 ± 5.63^{Ba}	25.1 ± 3.63^{Ba}	16.76 ± 1.85^{Ca}	0.2 ± 0.04^{Aa}	126.09 ± 30.85^{Bb}	121 ± 21.05^{BCb}	85.2 ± 7.99^{Cb}	0.34 ± 0.07^{Ab}	239.74 ± 40.41^{Bc}	187.66 ± 17.61^{Bc}	204.25 ± 9.33^{Bc}
Ca	587.41 ± 25.46^{ab}	567.83 ± 100.75^{a}	510.5 ± 32.85^{a}	524.69 ± 56.57^{a}	514.34 ± 42.18^{a}	476.02 ± 51.37^{a}	495.99 ± 34.91^{a}	477.44 ± 40.28^{a}	673.2 ± 88.97^{b}	562.08 ± 56.02^{a}	594.09 ± 72.02^{a}	553.64 ± 60.59^{a}
K	279.55 ± 8.02^{a}	284.86 ± 14.07^{a}	277.03 ± 8.46^{a}	264.12 ± 5.56^{a}	296.24 ± 7.94^{a}	270.58 ± 27.33^{a}	285 ± 13.53^{a}	284.53 ± 7.7^{ab}	301.01 ± 11.64^{a}	285.97 ± 23.95^{a}	283.13 ± 16.72^{a}	307.75 ± 9.69^{b}
Mg	49.4 ± 1.62^{Aa}	54.07 ± 3.74^{Aa}	50.11 ± 1.78^{Aa}	50.4 ± 3.75^{Aa}	48.37 ± 4.24^{Aa}	49.39 ± 2.73^{Aa}	50.31 ± 2.78^{Aa}	51.07 ± 2.04^{Aa}	60.94 ± 4.49^{Ab}	53.81 ± 5.16^{ABa}	50.77 ± 3.87^{Ba}	55.51 ± 4.72^{ABa}
Na	307.06 ± 20.66	308.66 ± 13.92	318.02 ± 11.94	312.09 ± 12.78	323.56 ± 37.72	310.54 ± 46.36	347.33 ± 12.37	348.78 ± 33.16	334.71 ± 23.05	323.66 ± 35.23	310.03 ± 31.43	334.65 ± 9.88
Liver												
Zn	5.58 ± 1.18	4.53 ± 0.75	4.57 ± 0.65	4.98 ± 1.03	5 ± 0.71	5.48 ± 1.33	4.97 ± 0.99	5.04 ± 1.27	4.95 ± 0.73	4.75 ± 1.51	4.82 ± 0.48	5.95 ± 1.02
Cd	2.16 ± 0.2^{Aa}	13.78 ± 2.16^{Ba}	11.73 ± 0.96^{Ba}	7.55 ± 0.9^{Ca}	1.95 ± 0.33^{Aa}	16.89 ± 1.16^{Ba}	12.98 ± 1.11^{Ca}	12.09 ± 2.44^{Cb}	2.49 ± 0.55^{Aa}	32.55 ± 1.64^{Bb}	26.4 ± 2.96^{Cb}	22.74 ± 1.88^{Dc}
Ca	5.43 ± 2	5.32 ± 2.66	3.38 ± 0.14	3.1 ± 0.32	3.84 ± 0.27	3.83 ± 0.36	3.9 ± 0.46	4.4 ± 2.02	4.34 ± 0.71	5.16 ± 1.49	4.04 ± 0.09	3.89 ± 0.25
K	277.86 ± 17.43	270.88 ± 12.55	268.73 ± 12.43	255.23 ± 35.07	275.84 ± 13.03	265.23 ± 19.7	256.03 ± 8.36	244.31 ± 29.64	270.77 ± 21.71	275.64 ± 12.12	272.62 ± 4.25	269.88 ± 11.42
Mg	41.14 ± 1.55	38.46 ± 2.45	38.99 ± 1.61	38 ± 3.48	40.12 ± 2.06	38.02 ± 1.58	36.55 ± 1.05	37.18 ± 5.46	38.51 ± 1.91	37.87 ± 1.58	37.02 ± 0.84	36.07 ± 1.44
Na	136.34 ± 16.52	142.38 ± 19.14	140.94 ± 3.98	139.61 ± 13.62	141.91 ± 9.42	139.26 ± 7.58	151.87 ± 15.76	148.23 ± 25.19	142.61 ± 9.34	148.04 ± 11.63	152.76 ± 6.99	157.71 ± 10.07
Brain												
Zn	1.12 ± 0.06	1.23 ± 0.08	1.2 ± 0.15	1.1 ± 0.17	1.22 ± 0.07	1.09 ± 0.14	1.31 ± 0.08	1.15 ± 0.05	1.18 ± 0.22	1.19 ± 0.03	1.14 ± 0.02	1.27 ± 0.1
Cd	BMQL ± BMQL	$0.92 \pm$ BMQL	BMQL ± BMQL	$0.65 \pm$ BMQL	BMQL ± BMQL	$0.84 \pm$ BMQL	BMQL ± BMQL	1.17 ± 0.68	BMQL ± BMQL	2.98 ± 2.39	3.69 ± 1.81	3.28 ± 2.46
Ca	12.43 ± 3.83	30.18 ± 18.54	96.08 ± 128.38	15.21 ± 7.88	15.68 ± 3.45	16.29 ± 5.84	108.34 ± 72.89	16.81 ± 5.92	83.95 ± 91.3	64.56 ± 72.84	12.73 ± 4	16.34 ± 5.62
K	404.35 ± 9.06	401.7 ± 11.27	398.73 ± 9.71	391 ± 10.06	399.48 ± 13.91	361.34 ± 48.32	397.15 ± 8.54	386.07 ± 7.67	399.54 ± 70.11	384.2 ± 13.74	383.85 ± 5.11	394.35 ± 14.79
Mg	31 ± 0.76	31.49 ± 1.22	30.61 ± 0.32	30.36 ± 0.64	29.42 ± 0.8	27.17 ± 3.61	29.83 ± 0.39	30.4 ± 0.72	30.32 ± 5.4	29.39 ± 0.99	29.66 ± 0.54	30.87 ± 1.58
Na	251.93 ± 15.12	241.61 ± 5.02	235.46 ± 7.11	238.72 ± 4.63	244.35 ± 4.08	223.94 ± 25.82	242.72 ± 8.39	238.92 ± 7.65	258.22 ± 52.08	255.3 ± 12.86	252 ± 8.24	248.58 ± 18.64
Muscle												
Zn	0.85 ± 0.27	0.8 ± 0.22	1.05 ± 0.44	0.71 ± 0.08	1.01 ± 0.13	1.1 ± 0.29	1.18 ± 0.46	0.95 ± 0.44	1.03 ± 0.57	0.91 ± 0.26	0.89 ± 0.21	0.83 ± 0.21
Cd	BMQL ± BMQL	BMQL ± BMQL	BMQL ± BMQL	BMQL ± BMQL	BMQL ± BMQL	BMQL ± BMQL	BMQL ± BMQL	BMQL ± BMQL	BMQL ± BMQL	BMQL ± BMQL	BMQL ± BMQL	BMQL ± BMQL
Ca	34.36 ± 15.05^{Aab}	32.15 ± 7.99^{Aa}	35.6 ± 5.56^{Aa}	36.6 ± 10.01^{Aa}	26.28 ± 3.17^{Aa}	29.56 ± 4.06^{ABa}	44.3 ± 11.14^{Ba}	30.55 ± 2.78^{ABa}	48.85 ± 9.93^{Ab}	31.22 ± 3.65^{ABa}	35.96 ± 5.42^{ABa}	30.31 ± 4.84^{Ba}
K	434.98 ± 20.03	405.59 ± 59.67	417.55 ± 25.65	425.6 ± 43.39	453.51 ± 17.26	438.93 ± 29.92	431.1 ± 27.76	445.5 ± 22.68	413.63 ± 63.63	419.94 ± 36.85	440.52 ± 22.42	430.05 ± 35.28
Mg	63.59 ± 2.68	58.78 ± 7.75	61.49 ± 2.14	64.7 ± 4.99	66.75 ± 2.3	64.83 ± 3.37	65.72 ± 1.95	68.68 ± 1.91	59.82 ± 8.86	63.07 ± 3.19	66.36 ± 1.14	62.83 ± 6.72
Na	83.35 ± 6.76^{a}	86.19 ± 17.5^{a}	92.27 ± 9.04^{a}	86.87 ± 4.5^{ab}	81.67 ± 6.29^{a}	83.32 ± 6.82^{a}	90.48 ± 11.9^{a}	102.2 ± 9.6^{a}	81.34 ± 19.43^{a}	85.16 ± 11.17^{a}	80.79 ± 2.12^{a}	79.18 ± 8.16^{b}
Carcass												
Zn	6.79 ± 0.23^{a}	7.3 ± 0.48^{a}	6.84 ± 0.78^{a}	6.33 ± 0.51^{a}	7.37 ± 0.54^{a}	6.98 ± 0.48^{a}	8.21 ± 1.4^{a}	7.94 ± 0.49^{b}	7.76 ± 0.6^{a}	7.61 ± 0.76^{a}	7.58 ± 0.35^{a}	8.9 ± 0.34^{b}
Cd	0.48 ± 0.08^{Aa}	2.31 ± 0.21^{Ba}	2.04 ± 0.41^{BCa}	1.47 ± 0.11^{Ca}	0.49 ± 0.09^{Aa}	5.2 ± 2.04^{Bb}	4.29 ± 1.11^{BCb}	3.16 ± 0.95^{Cb}	0.44 ± 0.06^{Aa}	8.61 ± 1.55^{Bc}	5.44 ± 1.03^{Cb}	6.53 ± 0.77^{BCc}
Ca	809.65 ± 64.35^{a}	851.89 ± 24.87^{a}	832.54 ± 100.62^{a}	813.25 ± 65.11^{a}	901.48 ± 69.22^{ab}	776.89 ± 119.67^{a}	888.19 ± 56.66^{a}	835.76 ± 180.48^{a}	1046.14 ± 132.91^{b}	948.89 ± 121.4^{a}	819.06 ± 147.31^{a}	977.97 ± 68.64^{a}
K	382.59 ± 7.52	391.08 ± 12.7	400.87 ± 29.1	400.54 ± 9.06	408.2 ± 16.86	386.26 ± 23.81	410.41 ± 10.74	402.73 ± 23.53	424.8 ± 25.36	407.37 ± 25.71	411.51 ± 22.25	423.69 ± 23.62
Mg	60.97 ± 1.32^{a}	61.79 ± 1.93^{a}	62.43 ± 5.11^{a}	62.55 ± 1.76^{a}	64.63 ± 3.85^{ab}	60.4 ± 3.38^{a}	64.65 ± 3.04^{a}	65.78 ± 4.97^{a}	71.79 ± 3.19^{b}	65.36 ± 5.25^{a}	66.13 ± 2.85^{a}	69.56 ± 4.94^{a}
Na	259.35 ± 8.85^{a}	268.34 ± 10.65^{a}	273.94 ± 24.63^{a}	276.67 ± 6.66^{a}	288.07 ± 14.95^{ab}	266.93 ± 15.96^{a}	292.12 ± 9.95^{a}	298.57 ± 30.65^{a}	314.82 ± 18.26^{b}	299.38 ± 20.34^{a}	286.66 ± 21.89^{a}	307.95 ± 22.33^{a}

SI-Table 8: Zinc (µmol/g dw), Cd (nmol/g dw) and electrolyte levels (µmol/g dw) in gills, liver, brain, muscle and carcass of *Cyprinus carpio* exposed to Zn_{fix}/Cd_{var} mixtures for 1, 3 and 7 days. Mean ± SD, N=5. Lower-case letters indicate significant differences ($p < 0.05$) of treatments among sampling days, capital letters indicate significant differences ($p < 0.05$) among treatments within the same sampling day.

	Day 1				Day 3				Day 7			
Gill	**Control**	**Zn_{fix}/Cd_{10}**	**Zn_{fix}/Cd_{25}**	**Zn_{fix}/Cd_{50}**	**Control**	**Zn_{fix}/Cd_{10}**	**Zn_{fix}/Cd_{25}**	**Zn_{fix}/Cd_{50}**	**Control**	**Zn_{fix}/Cd_{10}**	**Zn_{fix}/Cd_{25}**	**Zn_{fix}/Cd_{50}**
Zn	11.16 ± 0.77^{Aa}	10.32 ± 1.24^{Aa}	11.05 ± 0.91^{Aa}	10.32 ± 0.36^{Aa}	10.99 ± 0.5^{Aa}	11.82 ± 0.84^{ABa}	11.9 ± 1.07^{ABab}	13.84 ± 1.02^{Bb}	12.66 ± 0.95^{Aa}	15.26 ± 2.32^{Bb}	14.23 ± 1^{ABb}	15.75 ± 1.1^{Bb}
Cd	0.27 ± 0.04^{Aab}	13.31 ± 0.64^{Ba}	25.1 ± 3.63^{Ca}	43.3 ± 7.36^{Da}	0.2 ± 0.04^{Aab}	48.06 ± 3.5^{Bb}	121 ± 21.05^{Cb}	215.1 ± 24.02^{Db}	0.34 ± 0.07^{Ab}	91.91 ± 10.99^{Bc}	187.66 ± 17.61^{Cc}	360.96 ± 45.49^{Dc}
Ca	587.41 ± 25.46^{ab}	533.16 ± 78.42^{a}	510.5 ± 32.85^{a}	526.96 ± 56.65^{a}	514.34 ± 42.18^{a}	539.93 ± 37.66^{a}	495.99 ± 34.91^{a}	518.99 ± 67.43^{a}	673.2 ± 88.97^{b}	647.71 ± 45.15^{a}	594.09 ± 72.02^{a}	580.38 ± 83.69^{a}
K	279.55 ± 8.02^{a}	270.36 ± 9.55^{a}	277.03 ± 8.46^{a}	265.59 ± 15.01^{a}	296.24 ± 7.94^{a}	279.21 ± 11.18^{ab}	285 ± 13.53^{a}	289.48 ± 7.57^{ab}	301.01 ± 11.64^{a}	297.45 ± 12.44^{b}	283.13 ± 16.72^{a}	295.2 ± 7.54^{b}
Mg	49.4 ± 1.62^{Aa}	49.27 ± 2.25^{Aa}	50.11 ± 1.78^{Aa}	49.93 ± 2.58^{Aa}	48.37 ± 4.24^{Aa}	48.14 ± 2.81^{Aa}	50.31 ± 2.78^{Aa}	51.25 ± 2.83^{Aa}	60.94 ± 4.49^{Ab}	58.65 ± 3.56^{ACb}	50.77 ± 3.87^{Ba}	52.1 ± 4.62^{BCa}
Na	307.06 ± 20.66^{a}	311.83 ± 2.24^{a}	318.02 ± 11.94^{a}	299.03 ± 17.21^{a}	323.56 ± 37.72^{a}	321.12 ± 14.36^{a}	347.33 ± 12.37^{a}	344.35 ± 10.44^{b}	334.71 ± 23.05^{a}	319.97 ± 15.03^{a}	310.03 ± 31.43^{a}	325.01 ± 21.19^{ab}
Liver												
Zn	5.58 ± 1.18	4.78 ± 1.1	4.57 ± 0.65	4.95 ± 0.23	5 ± 0.71	4.46 ± 1.21	4.97 ± 0.99	5.84 ± 0.23	4.95 ± 0.73	5.28 ± 0.73	4.82 ± 0.48	5.74 ± 0.64
Cd	2.16 ± 0.2^{Aa}	4.62 ± 0.38^{Aa}	11.73 ± 0.96^{Ba}	17.31 ± 2.25^{Ca}	1.95 ± 0.33^{Aa}	6.21 ± 0.34^{Ba}	12.98 ± 1.11^{Ca}	27.03 ± 2.73^{Db}	2.49 ± 0.55^{Aa}	9.93 ± 0.72^{Bb}	26.4 ± 2.96^{Cb}	48.76 ± 3.13^{Dc}
Ca	5.43 ± 2	3.66 ± 0.12	3.38 ± 0.14	9.63 ± 9.47	3.84 ± 0.27	3.81 ± 0.35	3.9 ± 0.46	4.13 ± 0.34	4.34 ± 0.71	3.7 ± 0.17	4.04 ± 0.09	4.23 ± 0.24
K	277.86 ± 17.43	272.21 ± 15.94	268.73 ± 12.43	251.48 ± 24.75	275.84 ± 13.03	246.43 ± 31.09	256.03 ± 8.36	267.72 ± 14.4	270.77 ± 21.71	257.61 ± 14.92	272.62 ± 4.25	280.47 ± 6.88
Mg	41.14 ± 1.55^{a}	40.39 ± 2.04^{a}	38.99 ± 1.61^{a}	39.41 ± 2.73^{a}	40.12 ± 2.06^{a}	36.49 ± 3.33^{ab}	36.55 ± 1.05^{a}	36.69 ± 0.64^{a}	38.51 ± 1.91^{a}	34.7 ± 1.07^{b}	37.02 ± 0.84^{a}	36.58 ± 1.07^{a}
Na	136.34 ± 16.52	145.64 ± 9.92	140.94 ± 3.98	145.73 ± 16.09	141.91 ± 9.42	139.91 ± 9.42	151.87 ± 15.76	143.34 ± 14.4	142.61 ± 9.34	152.71 ± 8.66	152.76 ± 6.99	159.57 ± 7.9
Brain												
Zn	1.12 ± 0.06	1.14 ± 0.06	1.2 ± 0.15	1.32 ± 0.12	1.22 ± 0.07	1.19 ± 0.12	1.31 ± 0.08	1.15 ± 0.07	1.18 ± 0.22	1.18 ± 0.11	1.14 ± 0.02	1.14 ± 0.05
Cd	BMQL ± BMQL	BMQL ± BMQL	BMQL ± BMQL	0.8 ± 0.23	BMQL ± BMQL	0.52 ± BMQL	BMQL ± BMQL	2.81 ± BMQL	BMQL ± BMQL	1.32 ± 0.59	3.69 ± 1.81	1.94 ± 1.65
Ca	12.43 ± 3.83	11.45 ± 4.7	96.08 ± 128.38	33.36 ± 29.84	15.68 ± 3.45	24.46 ± 22.64	108.34 ± 72.89	107.3 ± 53.73	83.95 ± 91.3	23.1 ± 13.72	12.73 ± 4	31.22 ± 27.29
K	404.35 ± 9.06	395.99 ± 15.17	398.73 ± 9.71	377.93 ± 10.98	399.48 ± 13.91	390.96 ± 4.79	397.15 ± 8.54	387.75 ± 6.1	399.54 ± 70.11	388.72 ± 8.98	383.85 ± 5.11	390 ± 2.31
Mg	31 ± 0.76	30.86 ± 0.29	30.61 ± 0.32	30 ± 0.92	29.42 ± 0.8	29.89 ± 0.77	29.83 ± 0.39	30.17 ± 0.48	30.32 ± 5.4	30.2 ± 0.71	29.66 ± 0.54	30.4 ± 0.46
Na	251.93 ± 15.12	232.76 ± 9.01	235.46 ± 7.11	238.51 ± 6.67	244.35 ± 4.08	242.58 ± 6.7	242.72 ± 8.39	241.89 ± 8.56	258.22 ± 52.08	244.31 ± 12.75	252 ± 8.24	235.07 ± 7.27
Muscle												
Zn	0.85 ± 0.27	0.89 ± 0.14	1.05 ± 0.44	0.68 ± 0.2	1.01 ± 0.13	0.95 ± 0.17	1.18 ± 0.46	0.93 ± 0.31	1.03 ± 0.57	0.84 ± 0.12	0.89 ± 0.21	0.99 ± 0.14
Cd	BMQL ± BMQL	BMQL ± BMQL	BMQL ± BMQL	BMQL ± BMQL	BMQL ± BMQL	BMQL ± BMQL	BMQL ± BMQL	BMQL ± BMQL	BMQL ± BMQL	BMQL ± BMQL	BMQL ± BMQL	BMQL ± BMQL
Ca	34.36 ± 15.05^{ab}	39.94 ± 8.06^{a}	35.6 ± 5.56^{a}	27.03 ± 7.78^{a}	26.28 ± 3.17^{a}	43.7 ± 20.13^{a}	44.3 ± 11.14^{a}	33.58 ± 6.46^{a}	48.85 ± 9.93^{b}	41.07 ± 8.37^{a}	35.96 ± 5.42^{a}	43.92 ± 10.87^{a}
K	434.98 ± 20.03	413.49 ± 19.97	417.55 ± 25.65	400.54 ± 74.52	453.51 ± 17.26	408.9 ± 18.92	431.1 ± 27.76	437.45 ± 19.94	413.63 ± 63.63	420.63 ± 25.48	440.52 ± 22.42	469.88 ± 15.85
Mg	63.59 ± 2.68	63.52 ± 3.93	61.49 ± 2.14	60.51 ± 12.5	66.75 ± 2.3	63.79 ± 3.14	65.72 ± 1.95	68.8 ± 2.93	59.82 ± 8.86	64.07 ± 3	66.36 ± 1.14	68.19 ± 1.09
Na	83.35 ± 6.76	82.84 ± 4.32	92.27 ± 9.04	79.48 ± 17.06	81.67 ± 6.29	80.18 ± 5.01	90.48 ± 11.9	89.74 ± 14.58	81.34 ± 19.43	79.27 ± 4.24	80.79 ± 2.12	88.6 ± 9.39
Carcass												
Zn	6.79 ± 0.23^{a}	6.47 ± 0.41^{a}	6.84 ± 0.78^{a}	6.56 ± 0.6^{a}	7.37 ± 0.54^{a}	7.42 ± 0.41^{a}	8.21 ± 1.4^{b}	7.45 ± 0.32^{ab}	7.76 ± 0.6^{a}	7.74 ± 0.27^{a}	7.58 ± 0.35^{ab}	8.01 ± 0.43^{b}
Cd	0.48 ± 0.08^{Aa}	1.12 ± 0.09^{Ba}	2.04 ± 0.41^{Ca}	2.72 ± 0.3^{Ca}	0.49 ± 0.09^{Aa}	1.94 ± 0.14^{Bb}	4.29 ± 1.11^{Cb}	7.05 ± 0.79^{Db}	0.44 ± 0.06^{Aa}	3.2 ± 0.17^{Bc}	5.44 ± 1.03^{Cb}	11.59 ± 0.76^{Dc}
Ca	809.65 ± 64.35^{Aa}	825.35 ± 44.78^{Aa}	832.54 ± 100.62^{Aa}	812.78 ± 88.27^{Aa}	901.48 ± 69.22^{Aab}	904.22 ± 60.24^{Aa}	888.19 ± 56.66^{Aa}	891.59 ± 74.8^{Aa}	1046.14 ± 132.91^{Ab}	1020.47 ± 94.36^{Aa}	819.06 ± 147.31^{Ba}	997.05 ± 102.99^{ABa}
K	382.59 ± 7.52	389.62 ± 9.21	400.87 ± 29.1	385.27 ± 29.73	408.2 ± 16.86	394.26 ± 19.25	410.41 ± 10.74	391.7 ± 20.64	424.8 ± 25.36	410.51 ± 14.48	411.51 ± 22.25	399.79 ± 12.57
Mg	60.97 ± 1.32^{a}	61.74 ± 1.92^{a}	62.43 ± 5.11^{a}	61.2 ± 5.6^{a}	64.63 ± 3.85^{ab}	63.5 ± 2.96^{a}	64.65 ± 3.04^{a}	64.08 ± 4.16^{a}	71.79 ± 3.19^{b}	68.73 ± 4.49^{a}	66.13 ± 2.85^{a}	66.68 ± 3.51^{a}
Na	259.35 ± 8.85^{a}	263.13 ± 4.17^{a}	273.94 ± 24.63^{a}	265.37 ± 22.69^{a}	288.07 ± 14.95^{ab}	276.36 ± 23.91^{a}	292.12 ± 9.95^{a}	281.6 ± 19.79^{a}	314.82 ± 18.26^{b}	301.24 ± 15.43^{a}	286.66 ± 21.89^{a}	292.71 ± 17.07^{a}

SI-Table 9*:* Relative target gene mRNA abundance in gills and liver of *Cyprinus carpio* exposed to Cd_{fix}/Zn_{var} mixtures for 1, 3 and 7 days. Mean ± SD, N=4. Lower-case letters in subscript indicate significant differences ($p < 0.05$) of treatments among sampling days, capital letters in superscript indicate significant differences ($p < 0.05$) among treatments within the same sampling day.

Gill	**Day 1**				**Day 3**				**Day 7**			
	Control	**Cd_{fix}/Zn_{10}**	**Cd_{fix}/Zd_{25}**	**Cd_{fix}/Zn_{50}**	**Control**	**Cd_{fix}/Zn_{10}**	**Cd_{fix}/Zd_{25}**	**Cd_{fix}/Zn_{50}**	**Control**	**Cd_{fix}/Zn_{10}**	**Cd_{fix}/Zd_{25}**	**Cd_{fix}/Zn_{50}**
MT	1.04 ± 0.36^{Aa}	2.78 ± 0.31^{ABa}	3.76 ± 0.76^{Ba}	3.52 ± 0.4^{Ba}	1.01 ± 0.15^{Aa}	4.17 ± 0.5^{Bab}	4.99 ± 1.05^{BCa}	6.35 ± 1.41^{Cb}	1.05 ± 0.36^{Aa}	4.8 ± 0.76^{Bb}	3.86 ± 1.14^{Ba}	5.67 ± 0.42^{Bb}
GR	1.03 ± 0.28	1.12 ± 0.12	1.23 ± 0.19	1.07 ± 0.16	1 ± 0.1	1.01 ± 0.15	0.99 ± 0.04	0.99 ± 0.08	1 ± 0.06	1.04 ± 0.07	1.18 ± 0.11	1.17 ± 0.05
GST	1.06 ± 0.46	0.87 ± 0.32	1.01 ± 0.29	0.87 ± 0.37	1.03 ± 0.27	1.11 ± 0.17	1.18 ± 0.16	1.16 ± 0.21	1.02 ± 0.21	1.45 ± 0.28	1.21 ± 0.33	1.26 ± 0.2
SOD	1.05 ± 0.38	0.85 ± 0.18	0.91 ± 0.17	0.77 ± 0.17	1 ± 0.07	0.95 ± 0.09	0.88 ± 0.02	0.97 ± 0.1	1.01 ± 0.13	0.99 ± 0.1	0.86 ± 0.09	1 ± 0.17
CAT	1.01 ± 0.19	0.96 ± 0.16	0.91 ± 0.07	1.12 ± 0.29	1 ± 0.08	1 ± 0.05	1.08 ± 0.04	1.14 ± 0.11	1.01 ± 0.13	0.99 ± 0.06	1.04 ± 0.05	1.14 ± 0.18
GPx	1.03 ± 0.32	0.97 ± 0.35	1.05 ± 0.06	1.23 ± 0.78	1.01 ± 0.14	1 ± 0.14	1.17 ± 0.12	1.25 ± 0.18	1 ± 0.11	1.13 ± 0.11	1.2 ± 0.07	0.92 ± 0.14
CASP	1.03 ± 0.3	0.84 ± 0.2	0.94 ± 0.24	0.94 ± 0.25	1 ± 0.11	0.99 ± 0.14	0.82 ± 0.07	1.17 ± 0.16	1.01 ± 0.18	1.02 ± 0.15	0.91 ± 0.14	1.01 ± 0.12
Liver												
MT	1.01 ± 0.2^{Aa}	0.74 ± 0.1^{Aa}	0.95 ± 0.46^{Aa}	1.39 ± 0.2^{Aa}	0.75 ± 0.11^{Aa}	1.39 ± 0.35^{ABa}	2.08 ± 0.3^{Bb}	5.27 ± 0.45^{Cb}	1.04 ± 0.37^{Aa}	1.38 ± 0.33^{Aa}	1.81 ± 0.78^{Aab}	10.77 ± 0.93^{Bc}
GR	1.01 ± 0.14	0.97 ± 0.26	1.05 ± 0.31	0.7 ± 0.15	1.06 ± 0.39	0.75 ± 0.17	0.84 ± 0.19	0.87 ± 0.23	1.02 ± 0.26	0.8 ± 0.21	0.89 ± 0.11	1.12 ± 0.21
GST	1.02 ± 0.2	0.99 ± 0.2	1.1 ± 0.52	1.16 ± 0.18	1.09 ± 0.55	1.37 ± 0.3	1.52 ± 0.43	1.03 ± 0.23	1.02 ± 0.21	0.93 ± 0.57	1.29 ± 0.42	0.99 ± 0.23
SOD	1 ± 0.04	0.9 ± 0.16	0.83 ± 0.3	0.89 ± 0.06	1.02 ± 0.25	1.09 ± 0.22	1 ± 0.19	1.15 ± 0.25	1 ± 0.06	1 ± 0.09	0.87 ± 0.15	0.82 ± 0.15
CAT	1 ± 0.08	1.03 ± 0.15	1.01 ± 0.37	0.95 ± 0.09	1.01 ± 0.19	1.04 ± 0.17	1.16 ± 0.22	1.19 ± 0.17	1.01 ± 0.13	0.86 ± 0.15	0.96 ± 0.18	0.86 ± 0.12
GPx	1 ± 0.09	1.1 ± 0.09	1.05 ± 0.34	1 ± 0.3	1.03 ± 0.29	0.68 ± 0.14	0.73 ± 0.24	0.92 ± 0.18	1.01 ± 0.13	1.02 ± 0.17	0.89 ± 0.16	1.34 ± 0.1
CASP	1.02 ± 0.21^{Aa}	1.09 ± 0.22^{Aab}	1.36 ± 0.34^{Aab}	1.06 ± 0.41^{Aa}	1.02 ± 0.21^{Aa}	0.7 ± 0.21^{Aa}	0.84 ± 0.06^{Aa}	0.83 ± 0.27^{Aa}	1 ± 0.07^{Aa}	1.39 ± 0.17^{ABb}	1.62 ± 0.35^{Bb}	1.71 ± 0.2^{Bb}

SI-Table 10: Relative target gene mRNA abundance in gills and liver of *Cyprinus carpio* exposed to Zn_{fix}/Cd_{var} mixtures for 1, 3 and 7 days. Mean ± SD, N=4. Lower-case letters in subscript indicate significant differences ($p < 0.05$) of treatments among sampling days, capital letters in superscript indicate significant differences ($p < 0.05$) among treatments with in the same sampling day.

Gill	Day 1				Day 3				Day 7			
	Control	**Zn_{fix}/Cd_{10}**	**Zn_{fix}/Cd_{25}**	**Zn_{fix}/Cd_{50}**	**Control**	**Zn_{fix}/Cd_{10}**	**Zn_{fix}/Cd_{25}**	**Zn_{fix}/Cd_{50}**	**Control**	**Zn_{fix}/Cd_{10}**	**Zn_{fix}/Cd_{25}**	**Zn_{fix}/Cd_{50}**
MT	1 ± 0.07^{Aa}	4.59 ± 1.06^{Ba}	4.27 ± 0.58^{Ba}	3.75 ± 1.09^{Ba}	1.01 ± 0.17^{Aa}	3.89 ± 0.8^{Ba}	5.33 ± 1.09^{Ba}	4.95 ± 0.47^{Ba}	1.03 ± 0.28^{Aa}	2.85 ± 0.9^{ABa}	3.74 ± 0.95^{Ba}	3.72 ± 1.11^{Ba}
GR	1 ± 0.05^{A}	1.12 ± 0.1^{A}	1.13 ± 0.18^{A}	1.23 ± 0.09^{A}	1 ± 0.12^{A}	0.87 ± 0.11^{A}	0.97 ± 0.07^{A}	1.13 ± 0.12^{A}	1 ± 0.03^{A}	1.04 ± 0.07^{AB}	1.17 ± 0.12^{AB}	1.27 ± 0.13^{B}
GST	1.01 ± 0.15	1.07 ± 0.19	1.06 ± 0.3	1.04 ± 0.27	1.03 ± 0.26	0.88 ± 0.26	1.12 ± 0.11	1.04 ± 0.24	1.01 ± 0.17	1.07 ± 0.09	1.13 ± 0.32	1.02 ± 0.24
SOD	1.01 ± 0.14	1.04 ± 0.15	1.01 ± 0.14	1.03 ± 0.26	1 ± 0.11	0.85 ± 0.07	0.9 ± 0.07	0.91 ± 0.1	1 ± 0.11	0.85 ± 0.05	0.85 ± 0.13	0.8 ± 0.1
CAT	1 ± 0.1	1.1 ± 0.14	1.02 ± 0.08	1.1 ± 0.04	1 ± 0.07	1.07 ± 0.06	1.08 ± 0.03	1.1 ± 0.07	1 ± 0.06	1.08 ± 0.14	0.96 ± 0.06	1.01 ± 0.05
GPx	1.01 ± 0.18^{A}	1.12 ± 0.01^{A}	1.19 ± 0.1^{A}	1.07 ± 0.1^{A}	1 ± 0.06^{A}	0.94 ± 0.08^{A}	1.2 ± 0.07^{A}	1.19 ± 0.15^{A}	1.01 ± 0.13^{AB}	0.91 ± 0.05^{A}	1.11 ± 0.06^{AB}	1.22 ± 0.18^{B}
CASP	1.01 ± 0.17	0.97 ± 0.12	1.09 ± 0.2	0.97 ± 0.27	1.01 ± 0.14	0.97 ± 0.17	0.99 ± 0.11	0.99 ± 0.07	1.02 ± 0.2	0.93 ± 0.21	0.91 ± 0.09	0.88 ± 0.09
Liver												
MT	1.04 ± 0.34^{Aa}	1.33 ± 0.33^{Aa}	0.87 ± 0.42^{Aa}	1.56 ± 0.3^{Aa}	1.13 ± 0.72^{Aa}	2.45 ± 1.03^{ABa}	2.08 ± 0.5^{ABb}	4.17 ± 0.55^{Bb}	1.03 ± 0.31^{Aa}	1.8 ± 0.24^{ABa}	1.86 ± 0.83^{ABab}	3.77 ± 2.02^{Bab}
GR	1.06 ± 0.43	0.93 ± 0.12	1.08 ± 0.17	1.14 ± 0.4	1.04 ± 0.32	0.99 ± 0.15	0.79 ± 0.18	1.14 ± 0.19	1.05 ± 0.43	0.95 ± 0.36	0.68 ± 0.18	0.76 ± 0.18
GST	1.00 ± 0.11^{a}	1.10 ± 0.36^{a}	1.11 ± 0.53^{a}	0.86 ± 0.38^{a}	1.04 ± 0.38^{a}	1.17 ± 0.31^{a}	1.35 ± 0.37^{a}	1.83 ± 0.18^{b}	1.03 ± 0.28^{a}	1.08 ± 0.64^{a}	1.127 ± 0.48^{a}	1.07 ± 0.33^{ab}
SOD	1 ± 0.04	0.89 ± 0.2	0.96 ± 0.36	0.87 ± 0.09	1.01 ± 0.18	1.07 ± 0.24	1 ± 0.16	1.14 ± 0.11	1 ± 0.1	0.87 ± 0.16	1.03 ± 0.25	1.04 ± 0.27
CAT	1 ± 0.09	1.06 ± 0.33	1.01 ± 0.42	0.99 ± 0.1	1 ± 0.12	1.09 ± 0.14	1.08 ± 0.17	1.3 ± 0.06	1 ± 0.09	1.09 ± 0.2	0.99 ± 0.21	1.1 ± 0.16
GPx	1.01 ± 0.17	1 ± 0.29	0.97 ± 0.36	1.27 ± 0.2	1.03 ± 0.26	0.87 ± 0.11	0.7 ± 0.09	0.78 ± 0.05	1.01 ± 0.16	1 ± 0.17	0.82 ± 0.15	0.84 ± 0.21
CASP	1.01 ± 0.13^{Aa}	1.08 ± 0.26^{Aa}	1.05 ± 0.24^{Aa}	1.31 ± 0.13^{Aab}	1.01 ± 0.17^{Aa}	1.02 ± 0.13^{Aa}	1.06 ± 0.09^{Aa}	1.05 ± 0.14^{Aa}	1.02 ± 0.23^{Aa}	1.7 ± 0.31^{Bb}	1.32 ± 0.2^{ABb}	1.77 ± 0.28^{Bb}

Chapter 5.

Investigating the effects of a sub-lethal metal mixture of Cu, Zn and Cd on bioaccumulation and ionoregulation in common carp, *Cyprinus carpio*.

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

Modified version of Aquatic Toxicology (2020); 218, 105363

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.

5.1. 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.

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 (Wilson and

Taylor 1993, De Boeck et al. 2001). 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.

We chose a sublethal mixture of Cu, Zn and Cd at a low concentrations (Cu: 0.08 µM; Cd: 0.02 µM and Zn: 3 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. 2020). 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 and Technology 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. 2019). 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.

5.2. Material and Methods

5.2.1. 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).

5.2.2. Experimental set up

Exposures were performed in duplicate and each consisted 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: 0.02 µM and Zn: 3 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 °C. 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 ± 0.0015 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 ± 0.01 µM (4.3 ± 0.6 µg/L), Zn 2.71 ± 0.81 µM (176.9 ± 52.8 µg/L) and Cd 0.03 ± 0.004 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. 2020). Metal speciation was calculated using Visual Minteq.

5.2.3. 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).

5.2.4. 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 like were 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 SI-table 3.

5.2.5. 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).

5.3. 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+} 0.005, Cd^{2+} 0.02 and Zn^{2+} 2.07. These values corresponded to 7% of Cu^{2+}, 76% Zn^{2+} and 85% Cd^{2+} of total Cu, Cd and Zn measured in the water. 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 7.

5.3.1. Metal accumulation

5.3.1.1. 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.

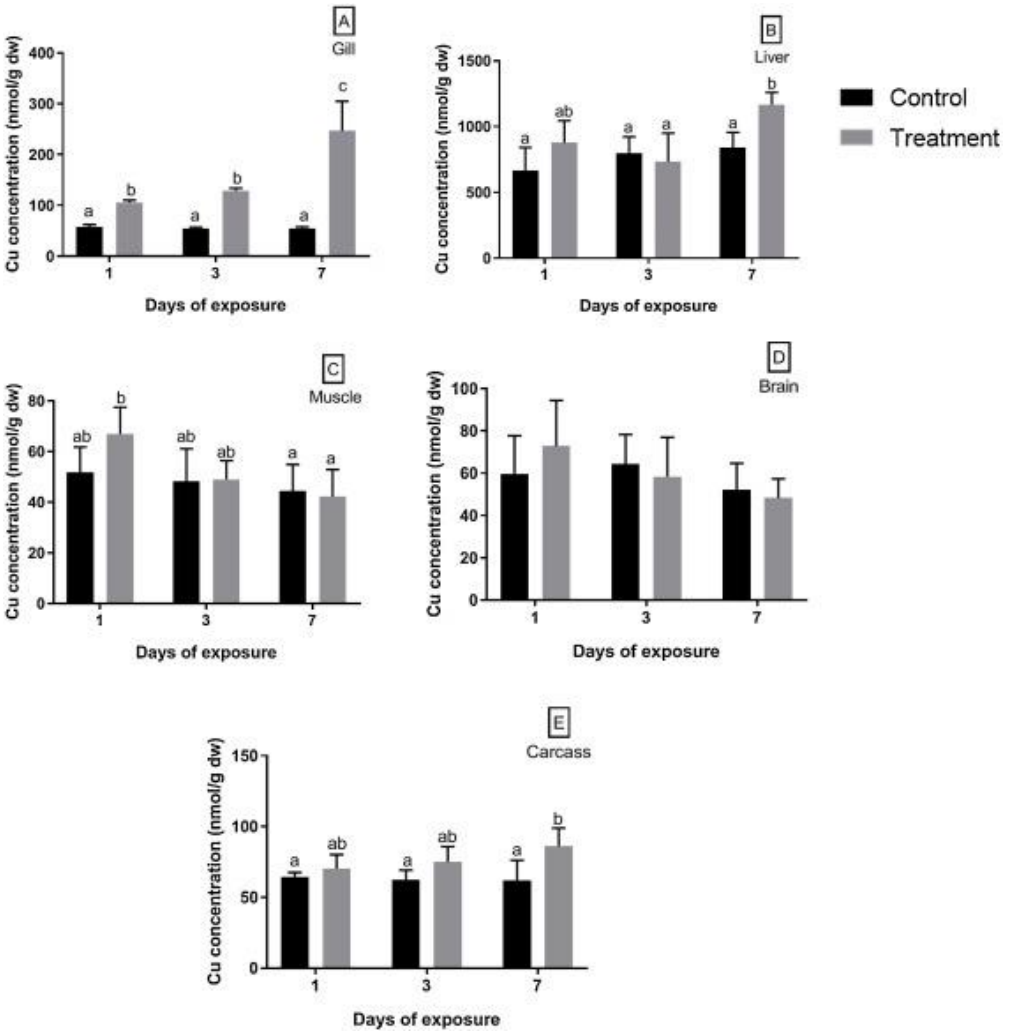


Figure 1: Copper concentration (nmo/g dw) in gills (A), liver (B), muscle (C), brain (D) and carcass (E) of *Cyprinus carpio* exposed to Cu/Cd/Zn mixture for 1, 3 and 7 days (mean ± standard deviation). Two-way analysis of variance (ANOVA) was performed followed by Tukey. Mean ± SD, N = 5, letters indicate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

5.3.1.2. Cd accumulation

Similar to Cu, Cd accumulation in the gills (Fig. 2A and SI-table 4) 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 6).

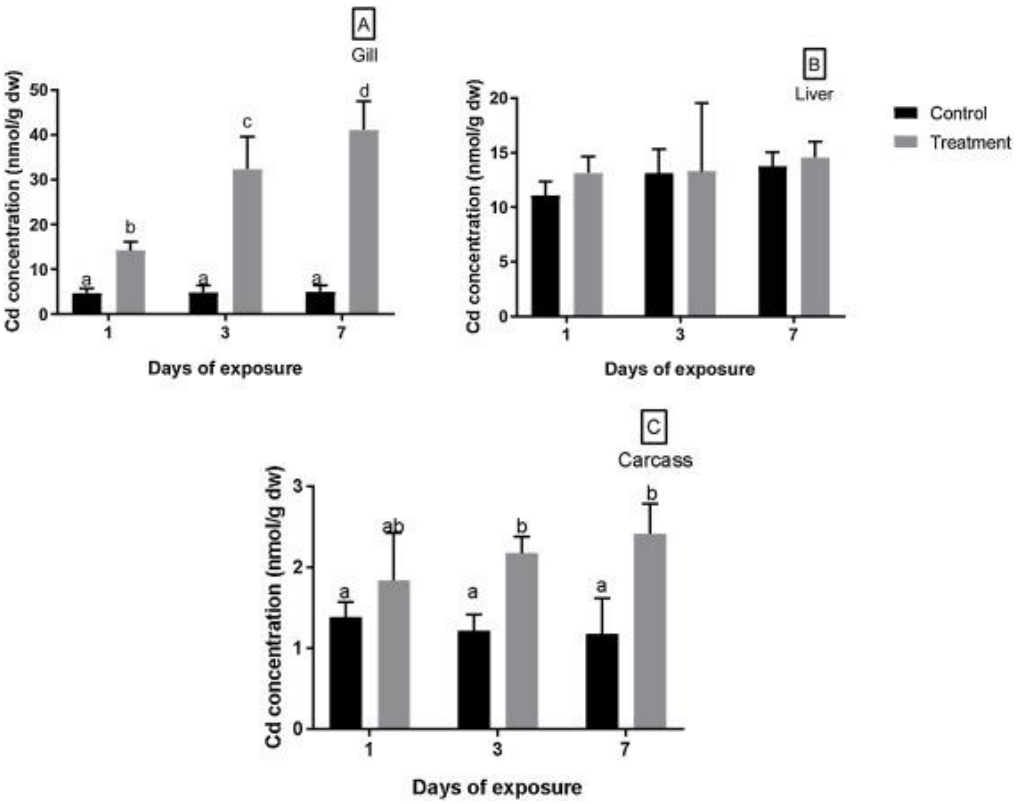


Figure 2: Cadmium concentration (nmol/g dw) in gills (A), liver (B), carcass (C) of *Cyprinus carpio* exposed to Cu/Cd/Zn mixture for 1, 3 and 7 days (mean ± standard deviation), results for muscle and brain were BMQL and are not showed. Two-way analysis of variance (ANOVA) was performed followed by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

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 4 and 6.

5.3.2. Electrolyte content

5.3.2.1. Sodium

Changes in Na homeostasis are shown in fig. 3. In the gills (Fig. 3A and SI-table 5), 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 6).

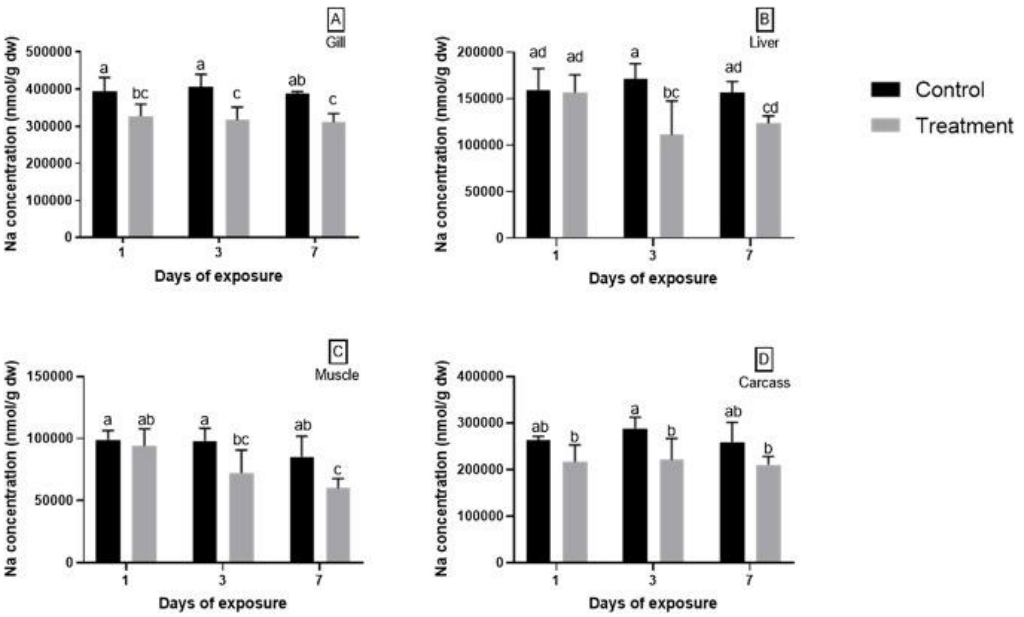


Figure 3: Sodium concentration (nmol/g dw) in gills (A), liver (B), muscle (C), carcass (D) of *Cyprinus carpio* exposed to Cu/Cd/Zn mixture for 1, 3 and 7 days (mean ± standard deviation). Two-way analysis of variance (ANOVA) was performed followed by Tukey. Mean ± SD, N = 5, letters indicate significant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

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 6) a decrease in K in the treatment compared to the control occurred at day 3 (≃ 13%). Magnesium content in the brain (see SI-table 6) 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 6).

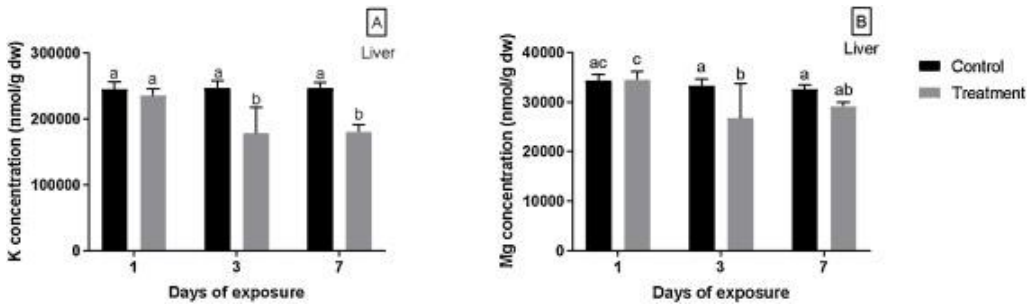


Figure 4: Liver potassium (A) and magnesium (B) concentration (nmol/g dw) of *Cyprinus carpio* exposed to Cu/Cd/Zn mixture for 1, 3 and 7 days (mean ± standard deviation). Two-way analysis of variance (ANOVA) was performed followed by Tukey. Mean ± SD, N = 5, letters indicate significant differences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

5.3.3. Gene expression

5.3.3.1. 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.

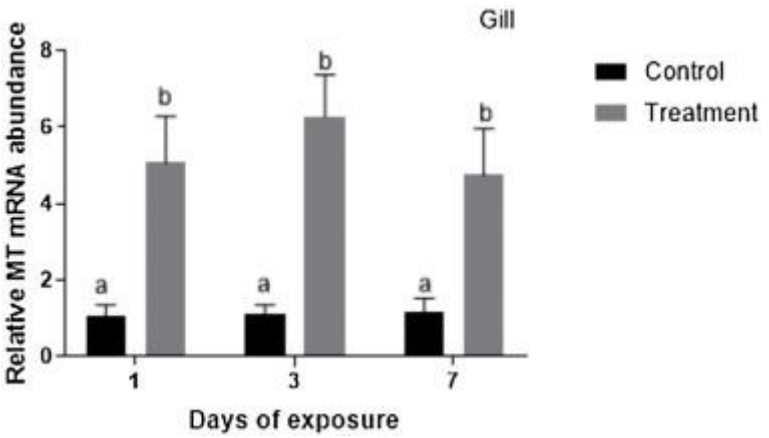


Figure 5: Relative genomic expression of gene coding for metallothionein in gills of *Cyprinus carpio* exposed to Cu/Cd/Zn mixture for 1,3 and 7 days (mean ± standard deviation). Two-way analysis of variance (ANOVA) was performed followed by Tukey. Mean ± SD, N = 6, letters indicate significant differences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

5.3.3.2. 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.

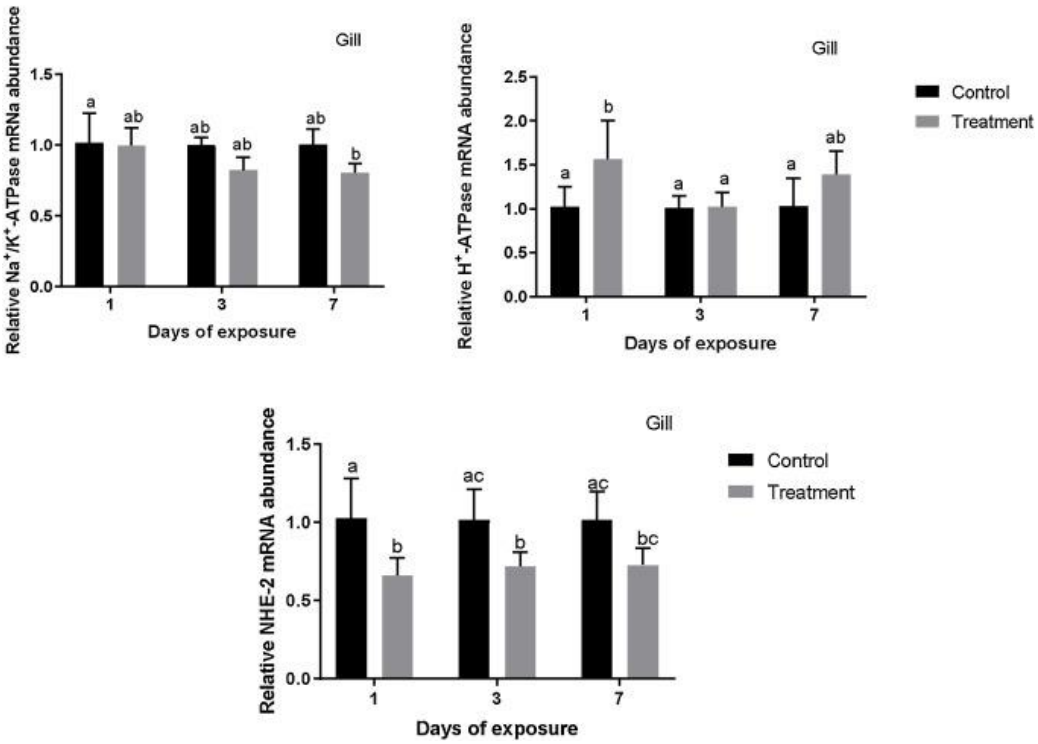


Figure 6: Relative genomic expression of gene coding for Na^+/K^+-ATPase, H^+-ATPase and NHE-2 in gills of *Cyprinus carpio* exposed to Cu/Cd/Zn mixture for 1,3 and 7 days (mean ± standard deviation). Two-way analysis of variance (ANOVA) was performed followed by Tukey. Mean ± SD, N = 6, letters indicate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

5.4. Discussion

As previously mentioned we hypothesized that metal bioaccumulation and induction of protective mechanisms such as MT would occur. 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.

5.4.1. 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 nmol/g dw for Zn. By extrapolating the results obtained from our own previous work (Castaldo et al. 2020a) 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 trace elements in the body and it has a key role for the activity of thousands 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. 2002b). 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. 2010a). However, MT induction is not always sufficient as Zhu et al. (2018a) 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.

5.4.2. 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.

5.5. 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, Čelechovská 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.

Acknowledgment

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

5.6. Supplementary information

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

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	

SI-Table 4: 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 5: 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 6: 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

Chapter 6.

Temperature effects during a sublethal chronic metal mixture exposure on common carp (*Cyprinus carpio*).

Giovanni Castaldo*, Marion Pillet, Leen Ameryckx, Lieven Bervoets, Raewyn M. Town, Ronny Blust, Gudrun De Boeck

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

Modified version of Frontiers in physiology (2021); 12, 284

Abstract

The aquatic environment is the final sink of various pollutants including metals, which can pose a threat for aquatic organisms. Waterborne metal mixture toxicity might be influenced by environmental parameters such as the temperature. In the present study, common carp were exposed for 27 days to a ternary metal mixture of Cu, Zn and Cd at two different temperatures, 10 and 20 °C. The exposure concentrations represent 10% of the 96 h-LC_{50} (concentration lethal for the 50% of the population in 96 h) for each metal (nominal metal concentrations of Cu: 0.08 µM; Cd: 0.02 µM and Zn: 3 µM). Metal bioaccumulation and toxicity as well as changes in the gene expression of proteins responsible for ionoregulation and induction of defensive responses were investigated. Furthermore the hepatosomatic index and condition factor were measured as crude indication of overall health and energy reserves. The obtained results showed a rapid Cu and Cd increase in the gills at both temperatures. Cadmium accumulation was higher at 20 °C compared to 10 °C , whereas Cu and Zn accumulation was not, suggesting that at 20 °C, fish had more efficient depuration processes for Cu and Zn. Electrolyte (Ca, Mg, Na and K) levels were analyzed in different tissues (gills, liver, brain, muscle) and in the remaining carcasses. However no major electrolyte losses were observed. The toxic effect of the trace metal ion mixture on major ion uptake mechanisms may have been compensated by ion uptake from the food. Finally, the metal exposure triggered the upregulation of the metallothionein gene in the gills as defensive response for the organism. These results, show the ability of common carp to cope with these metal levels, at least under the condition used in this experiment.

Keywords: Cyprinus carpio, temperature, metal pollution, ion-homeostasis, ionoregulation, mixture stress

6.1. Introduction

Metal pollution, as a result of rapid industrialization and urbanization, is increasing in fresh water systems. Anthropogenic inputs, through mining activities, industries and urbanization play a major role in the release of metals in the aquatic environment (Sevcikova et al. 2011). Since metal ions can be easily bioaccumulated, and are non-biodegradable and potentially toxic to the aquatic biota, they are of great concern (Feng et al. 2015). Among metals, copper (Cu^{+}), zinc (Zn^{2+}) and cadmium (Cd^{2+}) are perhaps some of the most studied pollutants due to their ecological importance and adverse effects on the organisms. Copper (Cu^{+}) and zinc (Zn^{2+}) are considered essential trace metals and are necessary for many metabolic processes, but they can be toxic when present in excess (Bury et al. 2003, Stržak et al. 2014). Cadmium (Cd^{2+}) on the other hand is considered a non-essential element (McGeer et al. 2011). Due to their harmful effects on aquatic life, these metals are categorized as priority pollutants in many countries in the world (Grosell 2011, Hogstrand 2011, McGeer et al. 2011, Stržak et al. 2014).

Metal ions can be accumulated by organisms living in polluted areas via direct uptake through the gills or via food (Perera et al. 2015). Copper, Zn and Cd can be taken up via gills through several ion channels (Niyogi and Wood 2003). Copper can be taken up via the high-affinity copper transporter (CTR1), the divalent metal transporter (DMT1) (Mackenzie et al. 2004, Grosell 2011, Komjarova and Bury 2014) or via a putative Na^{+}-channel, coupled with H^{+}-ATPase (Grosell 2011). Copper can impact Na^{+} homeostasis and interfere with ionoregulation affecting the activity of the Na^{+}/K^{+}-adenosine triphosphate (Na^{+}/K^{+}-ATPase) (Wilson and Taylor 1993, De Boeck et al. 2001). Zinc uptake occurs via the zinc transporter ZIP proteins (ZRT, IRT-like protein) and via the epithelial calcium channel (ECaC), competing in the latter case with Ca^{2+} for uptake (Bury et al. 2003, Zhao et al. 2014). Cadmium can be taken up through the divalent metal transporter (DMT1), and similar to Zn^{2+}, it can compete with Ca^{2+} for the uptake via the ECaC, affecting Ca^{2+} homeostasis (Bury et al. 2003, McGeer et al. 2011, Komjarova and Bury 2014).

One of the earliest findings in ecotoxicology is that temperature can affect tolerance to trace metals in ectotherms (Cairns et al. 1975). Changes in water temperature can occur as a consequence of anthropogenic activities or naturally, e.g. due to the day/night cycle or seasonal changes. The fish's body temperature is dependent on the ambient temperature, which is one of the most important physical factors for aquatic ectotherms (Brett 1971). Fish can respond to this alteration physiologically, via thermal adaptation and via behavioural thermoregulation (Ohlberger et al. 2008, Ward et al. 2010). Temperature can influence metabolism, osmoregulation, reproduction and behaviour (Fiess et al. 2007, Crossin et al. 2008, Vergauwen et al. 2010). Generally for metals, elevated temperatures above the optimal temperature range for the

organism, tend to enhance toxic effects of metals (Sokolova and Lannig 2008). Furthermore it has been suggested that an increased metabolic rate at elevated temperatures may contribute to metal accumulation (Sokolova and Lannig 2008). For instance, in several fish such as Nile tilapia (*Oreochromis niloticus*), zebrafish (*Danio rerio*) and gilthead sea bream (*Sparus aurata*) a higher accumulation of Zn (Karakoc and Dincer 2003, Guinot et al. 2012), Cd (Vergauwen et al. 2013) and Cu (Guinot et al. 2012) was reported with increasing temperature. Nevertheless, at higher temperatures, also depuration processes might be facilitated (Carvalho et al. 2004, Carvalho and Fernandes 2006). Several studies in fish, have reported that with increased temperature, also the toxicant accumulation (e.g. mercury, cadmium and arsenic) and depuration increased (Hodson and Sprague 1975, McGeachy and Dixon 1989, Nichols and Playle 2004). In addition studies on rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*) and stone loach (*Noemacheilus barbatulus*) showed that at higher temperatures the elimination rates of cesium (Cs) and Cd increased (Douben 1989, Ugedal et al. 1992, Cocchio et al. 1995, Nichols and Playle 2004).

Fish have defensive mechanisms involved in both essential metal-homeostasis and metal sequestration. Metallothioneins (MT) are low molecular weight metal binding proteins that are involved in the maintenance of Zn and Cu homeostasis (Hamilton and Mehrle 1986, Coyle et al. 2002, De Boeck et al. 2003). Metallothionein synthesis, although mainly linked with metal exposure, can be affected by other factors such as temperature (Van Cleef-Toedt et al. 2001, Guinot et al. 2012). For instance higher levels of MT proteins were found in sea bream liver when exposed to 30 °C in comparison to fish exposed at 22 or 27 °C. Similarly also in Nile tilapia MT levels increased as water temperature (20, 24, 28 or 32 °C) increased (Abdel-Tawwab and Wafeek 2014).

Although there are several studies describing the effects of metals and temperature in fish (Abdel-Tawwab and Wafeek 2017, Braz-Mota et al. 2017, Li et al. 2020), it is difficult to find information on metal mixtures combined with different temperature scenarios. In this study common carp (*Cyprinus carpio*) were exposed to a sublethal metal mixture of Cu, Zn, and Cd using nominal metal concentrations reflecting the 10% of the 96h-LC_{50} (the concentration that is lethal to 50% of the population in 96 h) previously determined in our lab by Delahaut et al. (2020) in fish kept at 20 °C. The used nominal concentrations were Cu: 0.08 µM; Cd: 0.02 µM and Zn: 3 µM which represents a mixture of metals at similar toxicity levels for each metal. Moreover, in Flanders (the Belgian region where this study was conducted), the limits for these metals are set to 0.11µM for Cu and 0.30 µM for Zn, whereas for dissolved Cd in rivers and lakes limits range (according to the water hardness) are between 0.004 to 0.013 µM (or 0.45 and 1.5 µg/L) (VLAREM II 2010). However, these limits can easily be exceeded as shown by Michiels et al. (2017), who reported dissolved metal

concentrations up to 0.95 µM, 0.05 µM, 52 µM for Cu, Cd and Zn, respectively in two different rivers. In addition a more recent study reported values ranging, from 0.02 to 0.04 µM for Cu, from 0.74 to 52 µM for Zn and from 0.004 to 0.06 µM for Cd over 5 different location in Belgium (Delahaut et al. 2019). Therefore the waterborne metal concentrations used in this study can be considered as environmentally relevant in Flanders.

The main aim of the present study was to understand to which extent different temperatures can affect metal mixture toxicity in common carp. This question will be answered by investigating metal bioaccumulation and MT gene expression as one of the defensive mechanisms in fish exposed to metals. Moreover changes in electrolyte levels, together with changes in the expression of genes involved in metal uptake and ion-homeostasis, that included CTR1, NHEs, Na^+/K^+-ATPase, H^+-ATPase were assessed. Finally, considering their ecological importance, integrative measures such as the hepatosomatic index (HSI) and condition factor (CF) were measured as well.

Based on previous results obtained in our lab (Castaldo et al. 2020c, Delahaut et al. 2020), we hypothesized on the one hand a fast Cu and Cd bioaccumulation, and on the other hand a delayed Zn accumulation occurring only after one week of exposure. Moreover we hypothesize that the presence of metals would trigger the response of MTs in order to mitigate possible deleterious effects. Concerning the electrolyte levels, as mentioned above, due to shared uptake routes between metal and electrolyte ions, a fast Na drop is hypothesized, together with a smaller Ca decrease. Furthermore, considering that a lower temperature leads to lower metabolic activity, we hypothesize that more pronounced effects would occur in fish exposed at 20 °C than in fish exposed at 10 °C. Finally, considering that the slope of the dose-response curves was similar for the metal ions (Delahaut et al., 2020) and that they were so steep that the 10% 96 h-LC_{50} used in our study could be considered sublethal, we anticipated that this mixture would remain sub-lethal.

6.2. Material and methods

6.2.1. Experimental model

Juvenile common carp, were obtained from Wageningen University (Netherlands). Fish were kept in 1000 L aquaria, filled with EPA (Environmental Protection Agency) medium hard water (Weber 1991) at 20 °C with a photoperiod of 12 h light and 12 h dark for several months. Prior to the start of the experiment fish were housed in three 200 L polyethylene tanks (PE) 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). Aeration and a biofilter were added to maintain water quality. Fish were divided into two series and kept in two different climate chambers (Weiss Technik, Belgium). The first series of fish was kept at 20 °C while in the second series the temperature of the climate chamber was reduced by 1 °C degree every three days until 10 °C was reached. The fish were acclimated for at least 2 weeks at the desired temperatures prior to the start of the experiment. Fish were fed with commercial food (Hikari® Staple™, Klundert, Netherlands) (minimum guaranteed crude protein and fat are 34% and 3%, respectively) to satiation once a day for the whole acclimation period. At the beginning of the experiment, in order to give the same amount of feed to the two experimental series, an amount of food representing the 2 % of the biomass was given to both the experimental series. This amount was adjusted once during the final weeks as the number of fish per tank decreased. After approximately 5 to 10 minutes food leftover was removed from each tank in order to reduce at minimum the metal absorption to the feed and consequent metal ingestion. 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)

6.2.2. Experimental set up

The same experimental set up and sampling method was used for both the experimental series, which ran in parallel. Experimental animals (N = 100 for each series) were sacrificed in order to collect samples. The fish, eight months old (length = 55 ± 9 and 57 ± 5 mm; weight = 2.7 ± 1.1 and 2.5 ± 0.6 g, respectively, at 10 and 20 °C), were divided between the two thermal exposure series (10 °C and 20 °C) in control and treatment tanks. Exposure tanks, for each series, consisted of three tanks for control and three PE tanks for the treatment. All the tanks were filled with 150 L of EPA medium-hard water as described above. Water parameters such as pH (8.2 ± 0.2) and conductivity (308 ± 2.5 µS/cm), were measured by the HQ30D Portable Multi-meter (Hach, USA). The dissolved organic carbon (DOC) (0.58 ± 0.23 ppm) was determined following the non-purgeable organic carbon (NPOC) protocol with the high-

temperature combustion, using non-dispersive infrared (HTC – NDIR). In each tank, oxygen was provided with an air stone. Water quality was ensured by a biofilter and checked daily. Water was changed when necessary in order to avoid the accumulation of ammonia and other waste products. Water samples were collected before and after water changes to ensure metal concentration stability with the 7700x ICP-MS (Agilent Technologies, Santa Clara, CA, USA). The measured control total metal concentrations in the 10 °C and 20 °C series were, respectively, 0.01 ± 0.001 and 0.01 ± 0.002 µM for Cu, whereas Zn and Cd remained below the quantification limit (BMQL). In the treatment during the experiment the total metal levels were for the 10 °C series: Cu 0.08 ± 0.02 µM, Zn 2.70 ± 0.42 µM and Cd 0.02 ± 0.003 µM. For the 20 °C series: Cu 0.08 ± 0.02 µM, Zn 2.32 ± 0.35 µM and Cd 0.02 ± 0.005 µM. Metal speciation for the two experimental series, calculated with the VMinteq software, using measured water parameters is shown in the supplementary information, SI-Tables 1 and 2. No abnormal fish behaviour was observed during the whole experiment.

6.2.3. Condition indices

Hepatosomatic Index was calculated according to the formula HSI= LW/BWx100, where BW is the body weight in g and LW is the total liver weight in g. The CF was calculated as CF= BW/L^3 where L= fish length in cm (Bervoets et al. 2009).

6.2.4. Metal accumulation and electrolyte levels in the tissues

At each sampling day (day 1, 7, 14, 21 and 27) ten fish from each treatment and each experimental series, 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). A muscle sample ≃ 22.4 ± 7.7 mg dry weight (dw) was cut near the caudal fin, the 1st and 4th gill arch of both left and right side were dissected and pooled per two fish to obtain sufficient tissue. The liver and the brain were collected and pooled per two fish. In addition the remaining carcasses per treatment and per sampling day were collected and pooled (two per sample) to have an overview of the whole body accumulation. The wet weight of the sampled tissues was recorded and the samples immediately frozen in liquid nitrogen and stored at -80 °C. Metal and electrolyte levels in gills, liver, muscle and brain were determined in five samples from each tissue (according to the pooled number of samples) at each sampling time and for each treatment. Samples, reference material (SRM-2976, Mussel tissue, National Institute of Standards and Technology, Gaithersburg, MD, USA) and blanks were collected in pre-weighed Eppendorf bullet tubes, dried for 48 hours at 60 °C and cooled in a desiccator for two hours. Subsequently the dry weight of the samples was recorded with a precision scale (Sartorius SE2, ultra microbalance). The digestion process was done as described by Reynders et al. (2006a) and Blust et al. (1988). Briefly, after a sample digestion of 12 h at room temperature using 69 % concentrated HNO_3 and three microwave steps, H_2O_2

was added to digest lipids and a further microwave digestion took place. Carcass samples (*N* = 5), collected in pre-weighed 50 ml Falcon tubes were processed similarly. The samples were digested using a hot block (Environmental Express, Charleston, SC, USA) for 30 min at 100 °C. At the end of the digestion process, all the samples were diluted using ultrapure Milli-Q (MQ), to reach a final acid volume concentration between 1 and 3 %.

Metal and electrolyte levels for the liver and carcasses were expressed not only as nmol/g dw or µmol/g dw, but also as nmol/tissue dw or µmol/tissue dw (calculated by multiplying the reported concentrations by the dw in g of the pooled tissue of interest) in order to take into account the effect of the temperature on liver size and the natural growth of the organism.

Metal and electrolyte levels were determined respectively, using a 7700x ICP-MS (Agilent Technologies, Santa Clara, CA, USA) and an iCAP 6300 Duo (Thermo Scientific, Waltham, MA, USA). Results obtained with the above instruments, refer to the total element, without considering the charges. Therefore, charges in the manuscript were added only when relevant for the discussion.

6.2.5. RNA extraction and real time PCR

The second and the third gill arch of individual fish and an aliquot of the pooled liver samples of two fish were used for total RNA extraction and gene expression. The RNA was extracted according to the manufacturer protocol from ~ 50 mg of tissue using Trizol (Invitrogen, Merelbeke, Belgium). RNA quantity and purity was determined with nano-Drop spectrophotometry (NanoDrop Technologies, Wilmington, DE), whereas the integrity was assessed with a 1 % agarose gel. The cDNA was synthesized according to RevertAid H minus First strand cDNA synthesis kit protocol (Thermo fisher, Fermentas, Cambridgeshire). According to the OD260/OD280 nm absorption ratio (higher than 1.8), four samples were selected and used for qPCR. The Real-time PCR was performed using the Brilliant III Ultra-Fast QPCR Master Mix (Agilent) for the Mx3000P QPCR System. The assay was performed following the Brilliant III Ultra-Fast QPCR Master Mix (Agilent) protocol for Agilent Mx3005P QPCR system in a final reaction volume of 20 µl. The reaction mixture contained 10 µl of Brilliant III Ultra-Fast QPCR Master Mix, 5.7 µl of sterile water, 500 nM of each primer,0.3 µl of reference dye and 5 ng of cDNA. The contamination of reagent was assessed including the “no template” control (e.g. sterile water) in the analysis. The general experimental run protocol as described by Shrivastava et al. (2017), consisted of a denaturation program (3 min at 95 °C), an amplification and quantification program repeated 40 times (15 seconds at 95 °C, 20 seconds at 60 ºC°C and a melting curve program (60 °C – 95 °C). Oligonucleotide primers were, β-actin (Wu et al. 2014), eEF (Sinha et al. 2012), H^+-

ATPase (Sinha et al. 2016), Na^+/K^+-ATPase (Castaldo et al. 2020c), Na+/H+ exchanger (Castaldo et al. 2020c), CTR1 (Castaldo et al. 2020b) and metallothionein (Reynders et al. 2006b). Quantification cycles (Cq) values were automatically calculated on the log curve for each gene with MxPro QPCR software (Agilent Technologies, Waldbronn, Germany). The stability of the reference genes was tested by two-ways ANOVA both in liver and in the gills. The presence of unique PCR product was assessed by means of the melting curve and the PCR product was verified on agarose gel. The primer efficiency was determined based on the slope of the standard curve. And the relative gene expression determined by means of the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). More primers information (e.g. sequence and efficiency) are given in SI-Table 3.

6.2.6. Statistical analysis

All data are presented as mean values ± S.D. For the statistical analyses, normality of the data was tested with the Shapiro-Wilk test and the homogeneity of the variance with the Levene test. If normality assumption was not met, data were log(y) or sqrt(y) transformed. Three-way analyses of variance (ANOVA), followed by Tukey test, were performed for the obtained data. The independent variables included in the analysis were temperature, dose and time. Data were considered statistically significant when p-value < 0.05. All statistical tests, with the exception of the Levene test, which was performed with R 3.6.0 software, were carried out with GraphPad Prism version 8.02 for Windows (GraphPad Software, La Jolla California USA). According to (Custer et al. 2000), for metal concentrations below the minimum quantification limit, a value of MQL/2 was assigned. If > 50 % of the observations were BMQL, no statistical tests were conducted. Data presented in the supplementary information, such as curve fitting the metal bioaccumulation as function of time, were analysed using the same software.

6.3. Results

6.3.1. Hepatosomatic index and condition factor

The HSI showed a marked difference, with higher values in all the groups of fish exposed at 10 °C as compared with fish exposed at 20 °C; no differences were observed between controls and treatments (Fig. 1). The CF in fish exposed at 10 °C was higher in all the control groups as compared with the same groups at 20 °C. From day 21 onward, also the treatment groups at 10 °C showed higher values as compared to fish exposed at 20 °C. However, no differences were observed between control and metal treated groups (Fig. 1).

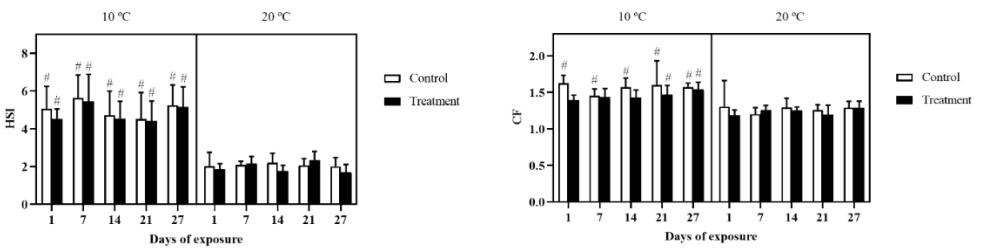


Figure 1: hepatosomatic index (HSI) and condition factor (CF), in common carp (*Cyprinus carpio*) exposed to a mixture of Cu, Zn and Cd for 27 days both at 10 °C and 20 °C. Mean ± SD, N = 10. The hash (#) indicates differences between the same groups (both control and metal treated) at different temperatures ($p < 0.05$).

6.3.2. Metal bioaccumulation

6.3.2.1. Metal accumulation in the gills

For fish exposed at 10 °C Cu content in fish gills (Fig. 2A) was higher in the treatment compared to the control for the whole duration of the experiment. In fish exposed at 20 °C Cu content increased significantly in the treatment at day 1, and peaked at day 7. After that, from day 14 onwards, a clear decreasing trend can be observed with values in metal exposed fish reaching control levels by the end of the experiment. Regarding the effect of the temperature, Cu levels in the control groups were stable within and between temperatures, whereas in the metal exposed groups from day 7 onwards, Cu content was always higher at 10 °C. Accumulation curves for Cu (SI-Fig. 1) showed a steady-state pattern for fish exposed at 10 °C, whereas for fish exposed at 20 °C a clear linear decreasing trend can be observed.

Concerning Zn accumulation, no statistical differences were observed between control and metal exposed groups in the analysed tissues (Fig. 2B). Zinc levels, both in the control and treatment groups remained constant in fish exposed at 10 °C, whereas in fish exposed at 20 °C, the treatment group accumulated more Zn at day 27 as compared to the beginning of the experiment. Regarding the effect of the temperature, the only difference occurred in the treatment group at day 21 for fish exposed at the lower temperature, which showed lower metal levels as compared with

the same group at 20 °C. Even though no differences occurred between controls and metal treatments, Zn accumulation curves showed a linear trend, which was more marked at 20 °C (SI-Fig. 1).

Cadmium accumulation in the gills (Fig. 2C) was always higher in the metal exposed fish as compared with the control groups in both the temperature scenarios. Over time, at 10 °C the control values showed some variation, with a difference occurring between day 21 and 27, whereas for the treated groups a continuous increase can be observed from day 1 onwards reaching the highest Cd content at day 27. In fish exposed at 20 °C the control groups at day 1 and 7 slightly varied compared to the same groups at different sampling days. In the treatment group, Cd content at day 14 was significantly higher compared with day 7, which was in turn higher compared to day 1. No differences occurred between the treatment groups at day 14, 21 and 27. In addition fish exposed to the metal mixture at 10 °C accumulated less Cd compared to fish exposed at 20 °C.

Cadmium accumulation showed an almost linear increase in both the exposure scenarios, without reaching a steady-state (SI-Fig. 1). As suggested also by the accumulation rates this was, in particular, true for fish exposed at 10 °C. The accumulation rates for fish exposed at the lower temperature remained constant through the whole experiment, whereas in fish exposed at 20 °C, the accumulation rates by the end of the experiment were around the 30 % lower compared to the large increase at day 1 (SI-Table 6).

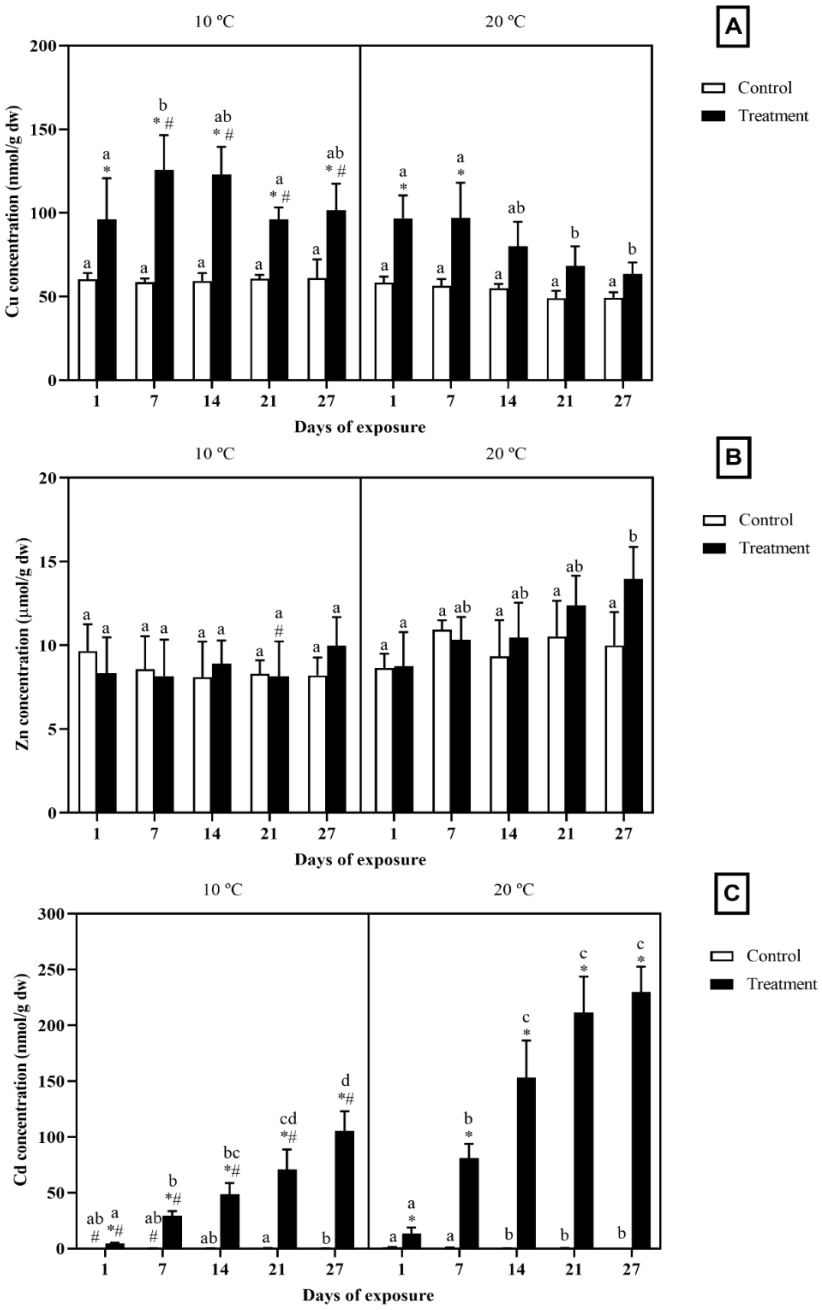


Figure 2: Gills (A) Cu (nmol/g dw), (B) Zn (µmol/g dw) and (C) Cd (nmol/g dw) accumulation in common carp exposed to a mixture of Cu, Zn and Cd for 27 days both at 10 °C and 20 °C. Mean ± SD, N = 5. Mean + SD. Asterisks (*) indicate differences between control and metal treatment at each sampling day, the hash (#) indicates differences between the same groups (both control and metal treated) at different temperatures and lowercase letters indicate significant differences of the same group among sampling days at each temperature ($p < 0.05$).

6.3.2.2. Metal bioaccumulation in the liver

Copper concentration in fish liver (Fig. 3.1A) did not show differences between control and metal exposed fish in either of the temperature scenarios, with the exception of the treatment group at day 14 at 20 °C. In fish exposed at 10 °C ºC, the Cu concentration for the control groups at day 1 and 7, and for the treatment groups at day 1, 7, 14 and 21 is lower as compared with the same groups of fish exposed at 20 °C. Regarding the differences over time, even though a slight increasing Cu trend can be observed at 10 °C, no differences occurred between the same treatments at different sampling points. In fish exposed at 20 °C the only difference can be observed between the control groups at day 1 and 14. Because liver sizes were considerably larger at 10 °C compared to 20 °C (see previous paragraph on HSI), we also reported absolute metal levels as metal content in the total liver (Fig. 3.2). For Cu content in the total liver (Fig. 3.2 A), no differences occurred between control and treatment in both the thermal profiles. However, in fish exposed at the lower temperature a more marked increasing trend can be observed with Cu levels becoming significantly higher in the final days of the experiment as compared to day 1. Moreover both the control and metal exposed groups at day 27 showed higher metal levels as compared with the same groups at 20 °C.

Zinc concentration in fish liver (Fig. 3.1B) showed no differences between control and metal treated fish in both the temperatures. For fish at 10 °C a constant Zn concentration can be observed, showing lower values for nearly all the groups compared to fish exposed at 20 °C. In fish exposed at 20 ºC the only difference over time can be observed between the treatment groups at day 7 and 27. When considering the total Zn content in the whole liver (Fig. 3. 2-B) similar to what observed for Cu, an increasing trend over time can be noticed in fish exposed at 10 °C, with both the control and treatment groups at the end of the experiment showing higher Zn values as compared with the same groups at day 1. No differences were observed between control and metal treated fish as well as for the differences between the two temperature scenarios.

Liver Cd concentration (Fig. 3.1C) showed no differences between control and treatment for fish exposed at 10 °C, whereas in fish exposed at 20 °C, higher Cd values in the treatment can be observed from day 7 onwards. In addition the cadmium concentration in fish exposed to the low temperature was always lower as compared with fish exposed at 20 °C. Regarding differences in time, for both the exposure scenarios, higher values were noticed in the treatment groups from day 14 onwards. Cadmium content in the whole liver (Fig. 3.2C) showed a similar trend to Cd expressed as nmol/g dw (Fig. 3.1C). However in fish exposed at 10 °C, the content in the treatment groups at day 7, 14 and 27 were significantly higher compared to the controls. Furthermore in fish exposed at 20 °C, differences between control and

treatment became significant from day 14 onwards. Differences according to the temperature can be observed in almost all the groups with higher values in fish exposed at 20 °C. At both temperatures, differences between the metal treated groups became more evident from day 7 and 14 onwards respectively, reaching the highest values by the end of the experiment.

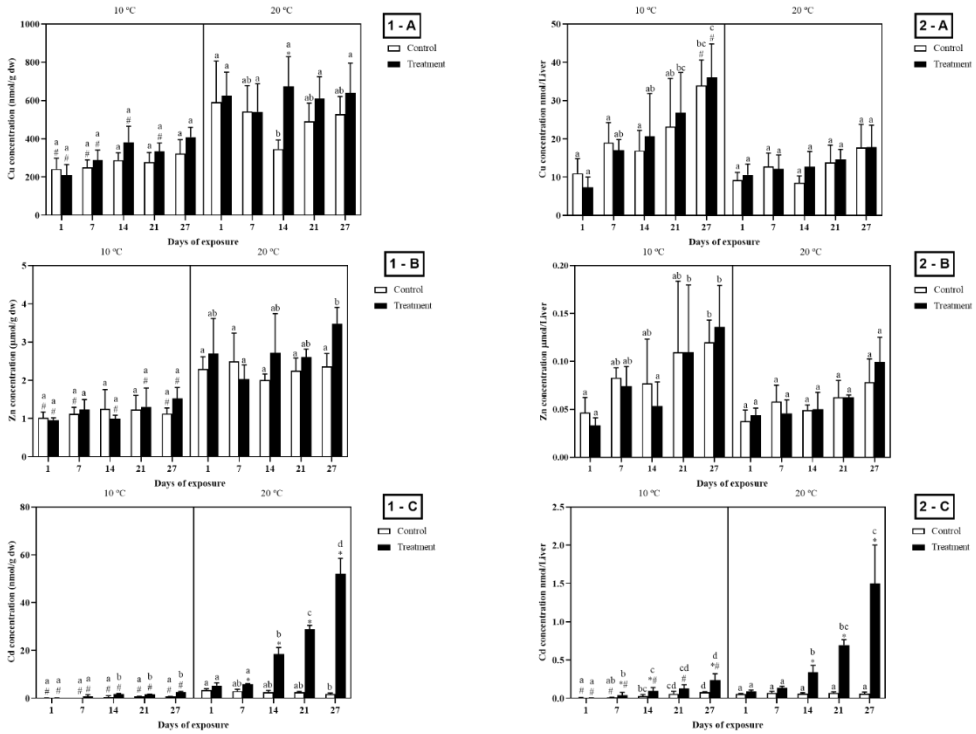


Figure 3: Cu (A-1), Zn (B-1), Cd (C-1) expressed as nmol/g dw or as µmol/g dw in the liver and Cu (A-2), Zn (B-2), Cd (C-2) expressed as total amount in nmol or µmol per liver in common carp exposed to a mixture of Cu, Zn and Cd for 27 days both at 10 °C and 20 °C . Mean + SD, N = 5. Mean ± SD. Asterisks (*) indicate differences between control and metal treatment at each sampling day, the hash (#) indicates differences between the same groups (both control and metal treated) at different temperatures and lowercase letters indicate significant differences of the same groups among sampling days at each temperature ($p < 0.05$).

6.3.2.3. Metal bioaccumulation in the other tissues

Metal levels in the brain (SI-Table 10), muscle (SI-Table 11) and in the remaining carcasses (SI-Table 12-13) are shown in the supplementary information. In the brain (SI-Table 10) no changes in Cu and Zn concentration were observed for either of the two exposure scenarios. Cadmium concentration in fish expose to 10 °C stayed below the detection limit in most of the analysed samples, whereas in the 20 °C scenario, was possible to detect the metal in all the treatment samples from day 14 onwards (SI-Table 10).

In the muscle (SI-Table 11) Cu content showed, in general, no differences between control and treatment groups. Nevertheless an increasing trend can be observed for fish exposed at 10 °C, with the highest values reached by the end of the experiment. Differences according to temperature can be observed in both the control and the treatment at day 27, with higher values at 10 °C. Regarding Zn, the only difference can be observed between the control group at day 1 in fish exposed to the low temperature, which had higher Zn concentration, compared with the same group in fish exposed at 20 °C. Cadmium concentration for fish exposed at 10 °C remained below the detection limit for the whole experiment, whereas in fish exposed at 20 °C it was possible to detect Cd in a few samples at day 14 and 21, and in all the samples at day 27. For more information see SI-material (SI-Table 11).

In the remaining carcasses (SI- Table 12), no differences were observed in Cu concentrations (nmol/g dw) between control and treatment groups either at 10 °C and 20 °C. Moreover the only difference occurring between the two temperatures can be noticed between the metal-exposed groups at day 1. In both the temperature scenarios a decreasing trend, with samples at day 27 showing the lowest Cu levels, can be observed in both the control and the metal treatment. Cadmium concentrations in the carcasses showed a rising trend both at 10 °C and 20 °C. However in fish exposed to the low temperature, differences between control and treatment can only be observed by the end of the experiment, whereas in fish exposed to 20 °C, they can be observed from day 1 onwards. Moreover in fish exposed at 10 °C, the treatment group at day 27 accumulated more Cd compared to the same group at day 1. In fish exposed at 20 °C the treatment groups at day 14 and 21 accumulated more Cd as compared to the same group at the beginning of the experiment. The only difference between the two exposure scenarios can be observed between the treatment groups at day 21, with fish exposed at the low temperature accumulating less metal.

6.3.3. Metallothionein gene expression

Gills MT gene expression (Fig. 4A) in fish exposed at 10 °C increased in the treatment as compared to the control from day 7 onwards, whereas in fish exposed at 20 °C this increase already started at day one and lasted until the end of the experiment. In both the exposure scenarios almost no differences according to time were noticed, with the exception of the treatment groups at day 27 as compared with the same groups at day 1 and 14 for fish exposed at the low temperature and day 7 for fish exposed at 20 °C. Regarding differences between the two temperature scenarios, the only noticeable dissimilarity can be observed between the treatment groups at day 1, with a slower start at 10 °C.

In the liver (Fig. 4B) no differences can be observed between control and metal-treatment or between the two temperature scenarios.

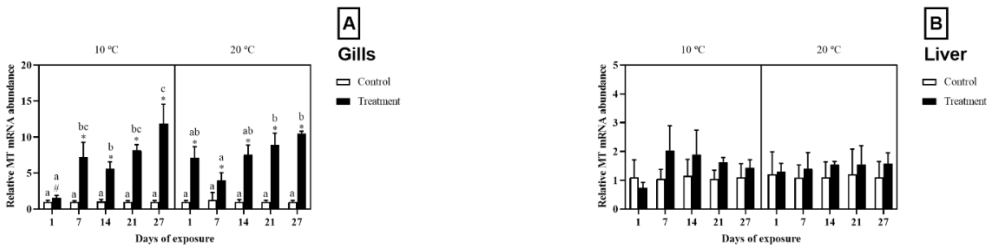


Figure 4: Relative metallothionein (MT) mRNA abundance in gills (A) and liver (B) of *Cyprinus carpio* exposed to Cu, Zn and Cd mixtures for 27 days. Mean ± SD, N = 4. Asterisks (*) indicate differences between control and metal treatment at each sampling day, the hash (#) indicate differences between the same groups (both control and metal treated) at different temperatures and lowercase letters indicate significant differences of the same group among sampling days at each temperature ($p < 0.05$).

6.3.4. Electrolyte levels

In the gills (SI-Table 7), K concentrations of fish exposed both at 10 °C and 20 °C did not show differences, except for the treatment group at day 21, which showed lower concentrations compared to the same group at day 7 in the 10 °C exposure scenario. Regarding Na content the only difference occurred between the control groups at day 1 and 7 and the metal exposed group at day 1, which showed lower values as compared with the same groups exposed at 20 °C. No differences were observed for Ca and Mg in either the temperature exposure scenarios.

In the liver, electrolyte concentrations expressed as µmol/g dw are shown in SI-Table 8. For K the only differences were observed between the two temperatures, with the control groups at day 1 and 7 and the metal exposed group at day 14 showing lower values in the 10 °C scenario. Similarly for Mg and Na all the groups at 10 °C showed lower values as compared with fish exposed at 20 °C. No differences were noticed for Ca.

In the brain (SI-Table 10), the only differences observed in the electrolytes concentrations occurred for K and Na. Regarding K, the day 21 control group of fish exposed at 10 °C showed a lower K concentration as compared with the same group exposed at 20 °C. Concerning Na, both the control and treatment from day 1 till day 21, showed lower electrolyte concentrations in fish exposed at 10 °C, compared with the 20 °C scenario.

In the muscle (SI-Table 11) no differences were noticed, except for Ca, which showed some variation at the low temperature.

In the remaining carcasses, electrolyte concentrations expressed as µmol/g dw are shown in SI-Table 12. For Ca no differences between control and treatment were observed. Furthermore at 10 °C, some variation occurred, leading to lower values compared to the start of the experiment, for the treatment at day 21. Similarly also in the 20 °C scenario, the control and the treatment groups at day 27 showed lower

values as compared with the previous sampling days. Furthermore, in the low temperature scenario, the treatment group at day 21, showed lower values compared to the group at 20 °C. Regarding K, Mg and Na, all three electrolyte showed lower concentrations at 10 °C as compared with the 20 °C. Moreover a slight decreasing trend, not always significant, could be observed over time both in fish exposed at 10 °C and 20 °C.

6.3.5. Expression of ion channels

The gene expression of the CTR1 in the gills (Fig. 5A) did not show differences between the control and metal treatment in fish exposed at 10 °C, whereas in fish exposed at 20 °C a significant increase can be observed in the treatment group at day 1. In fish exposed to the lowest temperature, the metal exposed groups at day 7 and 27 differ with the same groups at day 1. In the 20 °C exposure, the treatment at day 1 showed higher mRNA abundance as compared with the same groups at day 7, 14 and 21. The only differences between the two temperature scenarios occurred for the metal exposed group at day 1, with lower values in fish exposed at 10 °C.

For the expression of the H^+-ATPase (Fig. 5B) no differences were observed in fish exposed at 10 °C, whereas for fish exposed at 20 °C, the treatment group at day 27 showed a higher expression compared with the control group. Regarding the NHE (Fig. 5D), the only significant difference between control and treatment can be observed by the end of the experiment in fish exposed at the low temperature. Moreover, the metal treated group at day 27 showed a higher expression compared with the same groups at the previous sampling days. In fish exposed at 20 °C no differences occurred between control and treatment, even though an increasing trend for the treatment group over time can be seen. The Na^+/K^+-ATPase (Fig. 5C) did not show significant differences either for fish exposed at 10 °C or at 20 °C.

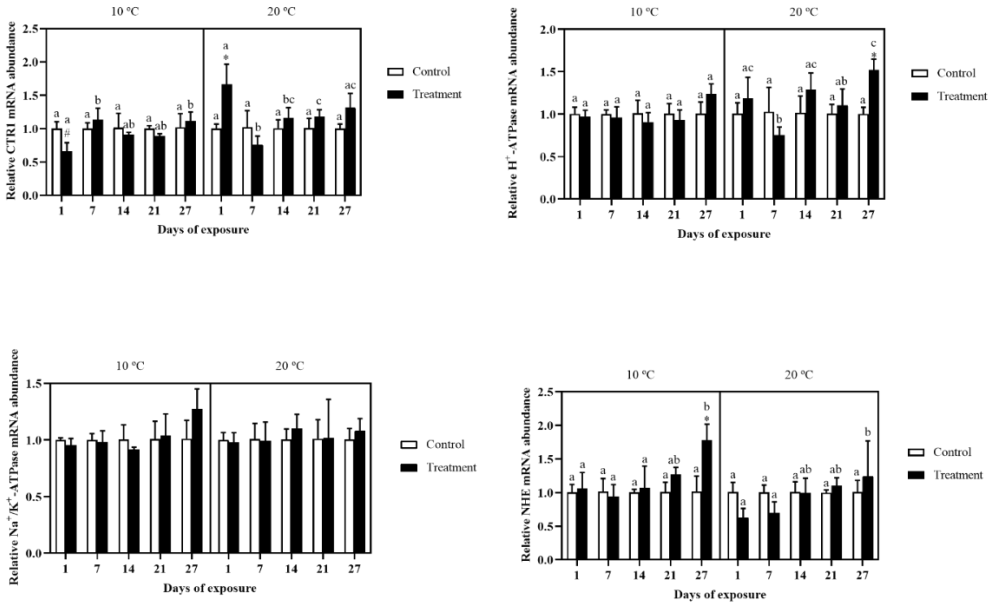


Figure 5: Relative copper transporter 1 (A), H^+-ATPase (B), Na^+/K^+-ATPase (C) and Na^+/H^+-exchanger (D) mRNA abundance in gills of *Cyprinus carpio* exposed to Cu, Zn and Cd mixtures for 27 days. Mean ± SD, N = 4. Asterisks (*) indicate differences between control and metal treatment at each sampling day, the hash (#) indicate differences between the same groups (both control and metal treated) at different temperatures and lowercase letters indicate significant differences of the same group among sampling days at each temperature ($p < 0.05$).

6.4. Discussion

6.4.1. Condition indices

The condition factor has frequently been used to express the overall health and wellbeing of fish (Bagenal 1978, Bolger and Connolly 1989, Bervoets and Blust 2003). In the present study, no differences in CF were reported between control and metal exposed fish either at 10 °C and 20 °C. Similarly, in previous studies using the gudgeon (*Gobio gobio*) or the rainbow trout, no relationship was found between environmental metal levels and CF (Dethloff et al. 2001, Bervoets and Blust 2003). Nevertheless, a marked difference can be observed between the two temperatures, with the CF being higher in fish exposed at 10 °C compared with fish exposed at 20 °C. Similar to the present finding, in zebrafish exposed to a chronic thermal scenario, the relative condition factor was lower in fish exposed to higher temperatures (32/34 °C) compared with fish kept in cold water (17/22 °C) (Pilehvar et al. 2019). Vergauwen et al. (2010) observed a decreased condition factor, which was in line with the energy stores, in zebrafish due to warm temperature (34 °C). At lower temperature, the reduced standard metabolic rate could have resulted in an increased energy availability for growth and thus an increased condition factor (Vergauwen et al. 2010).

The HSI, similar to what observed for the CF, was also affected by the temperature, with higher values in fish at 10 °C. This is in agreement with results obtained in rainbow trout, which showed a lower HSI in fish kept at 20 °C compared with fish kept at 11 °C (Sappal et al. 2015). Furthermore in the common carp, Fine et al. (1996) noticed and increased glycogen content in the liver and an increased HSI in fish kept at low temperature (17 °C) as compared to high temperatures (26 °C). Moreover, also in cold-acclimated trout a higher HSI was reported (Bouchard and Guderley 2003) indicating that energy reserves were stored in the liver (Voss 1985, Bouchard and Guderley 2003). On the other hand, this temperature effect was similar in control and metal exposed fish, which suggests that the metal load was not sufficiently high to increase stress and energy demand to an extent that would significantly deplete energy reserves.

6.4.2. Metal accumulation dynamics

In a simplistic view, which ignores the effects of temperature on repair mechanisms, it can be proposed that metal toxicity increase with the increase of temperature. For example, elevated metal toxicity at higher temperatures can be related to an increased uptake rate and thus higher metal levels in the organism. Additionally, elevated metabolic rates result in higher energy demands, and as consequence in elevated ventilation and feeding rates, which can lead to higher exposure to water and food contaminated by metals (Sokolova and Lannig 2008). However this is not always the case, as shown by some earlier studies, that reported no or reduced effects of increasing temperature on metal bioaccumulation and toxicity (Lannig et al. 2006,

Pereira et al. 2017). Five general toxicity patterns for pollutants have been proposed in a review by Sokolova and Lannig (2008), in which the type I and II are the most common patterns for trace metals. Both in type I and II the toxicity increases with an increasing temperature, however in the type I the toxicity increases only after a certain temperature threshold is reached.

6.4.2.1. Metal accumulation in the gills

One of the initial hypotheses was that metal accumulation, especially for Cu and Cd would occur fast in the gills at both the temperatures, but would be more pronounced in fish exposed at 20 °C. Both these assumptions were partially based on previous data obtained in our lab (Delahaut et al. 2020), as well as on the high conditional equilibrium constant, log *K*, for metal-gill binding sites. As reported by Playle (2004), in rainbow trout the conditional log *K*, values are 8.6, 7.4 and 5.6 (dm^3 mol^{-1}) for Cd, Cu and Zn, respectively. Therefore Cd binds about 16 times stronger to gills than Cu and 1000 times stronger than Zn.

Zinc, Cu and Cd accumulation in the gills, showed different accumulation trends. Zinc did not accumulate at all during the whole exposure period, whereas for Cu and Cd the accumulation started fast but to a different extent in the two thermal scenario. The lack of Zn accumulation in this experiment can be related to the fact that Zn, being one of the most abundant ions in the body, is strictly regulated (Zhao et al. 2014). For example in rainbow trout exposed to 234 µg/l (≃ 3.57 µM) of Zn, the accumulation of Zn started only after 10 days (McGeer et al. 2000a). Moreover Lange et al. (2002) found a Zn elevation of 1.4-fold in the treated group with 1000 µg/l (≃ 15 µM) of Zn, relative to the control only after 28 days of exposure. Therefore one can assume that the lack of Zn accumulation is more due to the regulation processes rather than to the fact that it was not taken up. Using data from Van Ginneken et al. (1999), an estimated net Zn accumulation at 3 h of 0.61 µmol/g dw and and 0.56 µmol/g dw, respectively for fish at 10 °C and 20 °C can be obtained. This let us assume that the Zn uptake starts quite rapidly and just as rapidly is transferred or eliminated. Nevertheless an inhibitory effect of the metal mixture on Zn uptake can not be excluded. It is known that metals share uptake routes. For instance Cd^{2+} and Zn^{2+}, due to their chemical characteristic similarity can compete each other for their uptake (Brzóska and Moniuszko-Jakoniuk 2001) and for instance a Zn uptake inhibition by Cd was reported by Saibu et al. (2018). Nevertheless it is worth to mention that in this experiment the waterborne Cd and Cu levels were, respectively at least ≃ 116 and 29 times lower compared to Zn. Therefore the lack of Zn accumulation can not be related solely to inhibited uptake. Regarding Cu, it is possible to observe a counterintuitive trend, with accumulated values at 10 °C mostly higher than at 20 °C and consistently above control levels. Several studies in different species showed an increased metal content with increased temperature. For instance in zebrafish exposed to Cu and/or Cd, there was higher body metal load when

the fish were exposed to warmer temperatures (Pilehvar et al. 2019). The killifish (*Poecilia vivipara*) kept at 28 °C accumulated more Cu compared to fish kept at 22 °C when exposed to waterborne metal concentrations of 20 µg/l (≃ 0.31 µM). However at metal concentrations of 9 µg/l (≃ 0.14 µM), no differences occurred between the two thermal exposures (Zebral et al. 2019). Therefore, inherent temperature tolerance, time and metal ion exposure levels also seem to play an important role. The observation of a peak in Cu levels followed by a steady decrease thereafter was rather unexpected considering that previous studies showed a continuous Cu increase or at least a steady state in the gills. For instance, in Nile tilapia, gills exposed to 40 µg/l Cu (≃ 0.63 µM), showed a constant increase in metal load up to day 7, after which the levels stabilized only to increase again from day 14 till day 21 (Monteiro et al. 2005). In European eel, exposed to 12 µg/l Cu (≃ 0.19 µM), the total branchial Cu content was near a steady-state at day 3-6 (Grosell et al. 1998). Furthermore, common carp exposed to 1 µM of Cu for 28 days showed a peak Cu content in the gills, during the first 24 hours, followed by slightly lower levels thereafter (Hashemi et al. 2008b). The clear decreasing trend in the experiment indicates that the gills are only a temporary target organ for some trace metals (e.g. Cu and Zn), after which they are transferred through the blood stream to other tissues for storage and excretion (Grosell 2011, Kondera et al. 2014). Interestingly, this seems to be much less the case for the non-essential metal Cd which steadily accumulated at 20 °C. Therefore, these transport and depuration processes seem to be more efficient for the essential metals, with Zn levels not increasing at all. Findings in the literature showed that these depuration processes are facilitated at higher temperatures (Carvalho et al. 2004, Carvalho and Fernandes 2006). So it seems that the depuration processes at 20 °C could counteract and even exceed the Cu uptake rates at 20 °C, whereas in fish kept at 10 °C, the lower metabolic activity slowed down the transfer of the metal into other tissues, resulting in elevated metal loads in the gills. Finally, similar to our observation, such a fast accumulation for Cd was already reported in common carp exposed to a combined metal solution, representing $1/10^{th}$ of LC_{50} / 48, of chromium (Cr), nickel (Ni), Cd and lead (Pb) (≃ 96, 85, 45 and 24 µM of Cr, Ni, Cd and Pb, respectively or 5 ppm) (*T* not specified) (Vinodhini and Narayanan 2008). Similarly also in the juvenile olive flounder (*Paralichthys olivaceus*), exposed to several Cd concentrations, an increasing metal trend was observed in the gills during the whole 30 days Cd-exposure period at T = 23 °C (Kim et al. 2010). In the present study, temperature showed an effect with lower Cd values in fish exposed at 10 °C. Similar findings were already reported in the Japanese eel (*Anguilla japonica*), in which lower Cd levels were reported in the eel exposed at 15 ºC than at T = 25 – 30 °C (Yang and Chen 1996). In principle the bioavailability of metals might be affected by the temperature, thus at higher temperatures, higher levels of free metal ions might be expected. Nonetheless as shown by empirical data and models (e.g. the biotic ligand model and the free ion activity model), the temperature in environmentally relevant ranges has a minor effect on metal speciation

(Luoma 1983, Bervoets and Blust 1999, Hassler et al. 2004, Sokolova and Lannig 2008). Thus as suggested by Sokolova and Lannig (2008), in order to explain the role of the temperature on metal uptake and accumulation, it is important to consider the biological effect of the temperature. Therefore the different trends observed for the different metal accumulations are more likely to be related to the effects of the temperature on the metabolic processes.

In vertebrates several mechanisms are known to be involved in Cu homeostasis, including the CTR1 (Anni et al. 2019). The regulation of the CTR1 is often contradictory and unclear (Boyle et al. 2011) and can be influenced by several metal ions. For instance, in zebrafish gills exposed to 0.016 µM Cu an upregulation of this transporter occurred (Leung et al. 2014). Whereas in sea bream a downregulation of the CTR1 in the intestine was observed in response to a high Cu diet, but not during waterborne metal exposures (Minghetti et al. 2008). In zebrafish liver exposed to 2.5 and 5 mg/l (≃ 20, 40 mM) of Cd, an inhibitory effect on the CTR1 gene was observed (Zhu et al. 2018b). Similarly a decreased expression was observed in the liver of zebrafish exposed to 200 µg/l (≃ 1.77 µM) of Cd, with a further decrease observed when fish were exposed at 34 °C (Guo et al. 2018). In the present experiment the increase observed at day 1 for fish exposed at 20 °C, is counterintuitive from a primary defense mechanism point of view, i.e. a downregulation of the transporter would be expected in order to slow down metal accumulation. Moreover, considering the presence of Cd in the mixture, a decreased CTR1 gene expression could be expected, as demonstrated in zebrafish (Guo et al. 2018, Zhu et al. 2018b). It is known that cortisol, which plays a role in the up-regulation of CTR1 gene (Tellis et al. 2012), can increase in response to metal exposure (De Boeck et al. 2001). Therefore, the observed trend may be attributed to an unexpected stressful situation for the organism, such as the metal exposure, which increased temporarily cortisol levels, leading to temporary up-regulation of this gene. Nevertheless further studies are needed to validate this thought.

6.4.2.2. Metal accumulation in the remaining tissues

Metal accumulation in the remaining tissues showed a different pattern between essential metals and non-essential metals. Zinc and Cu levels in the metal exposed groups did not show any significant increase as compared to the control in any of the analysed tissues. For example, similar findings were already observed for Cu in common carp muscle and in rainbow trout brain (De Boeck et al. 1997, Shaw et al. 2012). This gives an indication that the liver storage capacity and excretion processes were able to cope with the metal concentrations. However for the non-essential metal Cd, the situation is different suggesting that at lower temperatures the lower metabolic activity can result in less efficient metal transfer, as supported by findings in the remaining carcasses. Secondly this gives an indication of the important role played

by the liver, in protecting more vulnerable organs from metal toxicity as previously observed in different fish species (Brown et al. 1986, Hollis et al. 2001, Reynders et al. 2006a). In the brain the general lack of metal accumulation is reasonable considering that this organ is protected by the blood brain barrier (Evans and Hastings 1992, Szebedinszky et al. 2001). Nevertheless Cd was detected in all the brain samples of fish exposed at 20 °C from day 14 onwards, suggesting that temperature and time are important factors for brain. From the obtained results, it seems that for a non-essential metal such as Cd, the temperature effect is marked for most tissues, with higher metal content at 20 °C due to the higher metabolism. Our study also highlights that differences in temperature and metabolic rate can have profound effects on condition indices and organ sizes. Therefore in studies taking into account multiple stressors, it is important when interpreting the results to consider the effect of ambient parameters (e.g. temperature) on the organismal fitness (e.g. liver size) which consequently might affect the outcome of the chemical stressor (e.g. metal bioaccumulation and depuration). In this way, dilution effects on metal content are not underestimated.

6.4.3. Metallothionein gene expression

Several studies have pointed out the relationship between metal accumulation and MT levels in different tissues, and the protective role played by this protein towards metal ion toxicity (De Smet et al. 2001, Lange et al. 2002, De Boeck et al. 2003, De Boeck et al. 2010a). However to the authors' knowledge only few studies have been conducted in order to understand the effects of both metals and temperature on MT synthesis in fish.

In the present experiment, the MT mRNA expression was nearly always increased in the gills, but not in the liver which accumulated relatively low metal levels and is known to have higher MT basal level (Hashemi et al. 2008b). The increased gene expression in the gills in response to metal accumulation is an appropriate response when metals accumulate. However further studies assessing post transcriptional events are needed to further validate this thought.

In this experiment, fish kept at 10 °C showed a slightly delayed response in the gills. A temperature-dependent induction kinetics of MT was also observed in rainbow trout Zn-exposed hepatocytes, showing that even small changes in temperature (from 9 °C to 6 °C) can have serious impacts on the kinetics of MT efficiency and induction (Hyllner et al. 1989). This can be related to a lower plasma membrane fluidity (Krasne et al. 1971), which in turn affects the ion channel permeability to the metal and subsequent MT mRNA induction (Van Cleef-Toedt et al. 2001).

6.4.4. Ionoregulation and electrolyte levels

It is well known that metals, such as Cu, Zn and Cd can compete with major electrolyte ions for uptake and thereby alter their physiological levels (Bury et al. 2003, De Boeck et al. 2010b, Grosell 2011, McGeer et al. 2011). In the present experiment no differences between metal-treated and control fish occurred in any of the analyzed tissues, for either thermal exposure profile. The relatively stable Na levels are surprising considering that gills are one of the most affected tissue as reported in several studies (De Boeck et al. 2001, Grosell et al. 2002, Grosell and Wood 2002, Mackenzie et al. 2004). Moreover this finding is in disagreement with a previous study from our lab on common carp using comparable metal concentrations and showing a Na loss from the first day of exposure (Castaldo et al. 2020c). However it is worth to mention that fish in the present work were fed. It has been demonstrated that in rainbow trout, the ingested Na^+ is absorbed within hours (Smith et al. 1995), thus the steady levels of Na and electrolytes in general might be related to the presence of food. This thought is supported by the few significant changes that occurred, in the expression of genes involved with Na^+-homeostasis. However further chronic studies, including not only gene expression data, but also protein levels and enzyme activity are needed to underline the mechanisms involved in this process.

Temperature changes are known to impair osmoregulatory ability in teleosts, such as common carp (Metz et al. 2003) and Mozambique tilapia (*Oreochromis mossambicus*) (Fiess et al. 2007). However in this experiment, the only trend was that electrolyte ion concentrations (mainly for Na, in the gills and in the brain) were lower at 10 °C. For example in the gills, reduced Na levels were observed during the first week of exposure. One has to take into account that fish were acclimated for at least two weeks at 10 °C before the start of the experiment, and this is the time that was needed for rainbow trout exposed to cold water to restore the Na^+ influx to earlier warm temperature levels (Gonzalez and McDonald 2000). Possibly, this adjustment of Na homeostasis took a slightly longer in common carp.

6.5. Conclusions

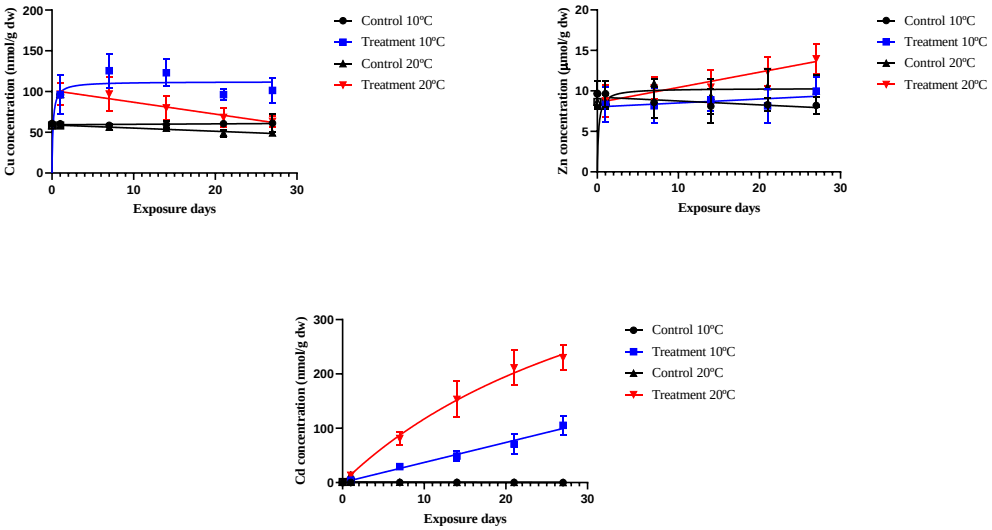
In the present experiment, the effects of a mixture of metals at two different temperatures on several endpoints were investigated in fish. Temperature had a considerable effect on condition indices, with higher values at 10°C, suggesting that a decreased metabolic rate led to an increased energy availability for growth and energy storage. As initially hypothesized, efficient regulation processes for Zn took place. In fact no accumulation was reported for this metal at all. Moreover, the results observed in the gills, let us speculate that the depuration and regulation processes for essential metals over a long exposure time were more efficient at 20 °C than at 10 °C, as suggested by the reduction of the initially elevated amount of accumulated Cu. As initially hypothesized, the metal accumulation in the gills triggered the induction of the

MT gene in order to mitigate possible deleterious effects caused by metal species. A hypothesis which was not confirmed by the present study was a fast loss of Na due to the metal mixture. Even though the aim of this work was not to investigate the role of feeding, in our case it is likely that the presence of the food masked the effects of metals on the electrolyte losses which we observed in earlier studies with unfed fish. Nevertheless, more studies focusing on the role of food in the ion homeostasis during metal exposure are needed. Overall, the data suggest that common carp is able handle this level of contaminants, at least under the exposure conditions of this experiment. Nonetheless, as suggested by Sokolova and Lannig (2008), more studies on the interaction of metal pollution together with natural stressors more representative of environmental conditions, such as fluctuating temperature and interaction with salinity and CO_2 on different levels of biological organization are needed to construct models to predict the effects of multiple stressors on ectotherm populations and to determine safety margins in ecological risk assessment.

Acknowledgments

We thank Steven Joosen and Karin Van den Bergh and for their technical assistance and we are grateful to the reviewers for their constructive comments.

6.6. Supplementary material



SI-Fig. 1: Gill Cu, Zn and Cd concentration over time. Dotted lines indicate assumed accumulation during the first day, extrapolating starting values from control values at day 1. The Michaelis-Menten (Y = Vmax*X/(Km + X)) curves and lines (Y= YIntercept + X*Slope) were fitted using measured metal concentration in the tissue.

SI-Table 1: Chemical speciation in the exposure media, for fish exposed at 10 °C calculated using nominal salt concentrations and measured metal levels with the equilibrium speciation code VMinteq.

Component	Concentration (mol/l)	% of total concentration	Species name
Cu^{2+}	2.9318E-09	3.665	Cu^{2+}
		6.988	$CuOH^{+}$
		0.027	$Cu(OH)_3^{-}$
		0.561	$Cu(OH)_2$ (aq)
		0.314	$CuSO_4$ (aq)
		86.674	$CuCO_3$ (aq)
		0.134	$CuHCO_3^{+}$
		1.635	$Cu(CO3)_2^{2-}$
Zn^{2+}	1.6675E-06	61.760	Zn^{2+}
		0.035	$Zn(CO_3)_2^{2-}$
		2.429	$ZnOH^{+}$
		14.988	$Zn(OH)_2$ (aq)
		5.326	$ZnSO_4$ (aq)
		0.042	$Zn(SO_4)_2^{2-}$
		14.274	$ZnCO_3$ (aq)
		1.133	$ZnHCO_3^{+}$
Cd^{2+}	1.6550E-08	82.749	Cd^{2+}
		0.264	$CdOH^{+}$
		0.286	$CdCl^{+}$
		7.249	$CdSO_4$ (aq)
		0.092	$Cd(SO4)_2^{2-}$
		1.521	$CdHCO_3^{+}$
		7.791	$CdCO_3$ (aq)
		0.040	$Cd(CO_3)_2^{2-}$

SI-Table 2: Chemical speciation in the exposure media, for fish exposed at 20 °C calculated using nominal salt concentrations and measured metal levels with the equilibrium speciation code VMinteq.

Component	Concentration (mol/l)	% of total concentration	Species name
Cu^{2+}	2.2136E-09	2.767	Cu^{2+}
		8.839	$CuOH^{+}$
		0.020	$Cu(OH)_3^{-}$
		1.626	$Cu(OH)_2$ (aq)
		0.265	$CuSO_4$ (aq)
		84.284	$CuCO_3$ (aq)
		0.131	$CuHCO_3^{+}$
		2.063	$Cu(CO3)_2^{2-}$
Zn^{2+}	1.3287E-06	57.273	Zn^{2+}
		0.054	$Zn(CO_3)_2^{2-}$
		5.042	$ZnOH^{+}$
		13.846	$Zn(OH)_2$ (aq)
		5.332	$ZnSO_4$ (aq)
		0.038	$Zn(SO_4)_2^{2-}$
		17.049	$ZnCO_3$ (aq)
		1.354	$ZnHCO_3^{+}$
Cd^{2+}	1.5929E-08	79.644	Cd^{2+}
		0.561	$CdOH^{+}$
		0.282	$CdCl^{+}$
		7.809	$CdSO_4$ (aq)
		0.087	$Cd(SO4)_2^{2-}$
		1.887	$CdHCO_3^{+}$
		9.658	$CdCO_3$ (aq)
		0.064	$Cd(CO_3)_2^{2-}$

SI-Table 3: Set of primers (F=forward; R=reverse) designed for common carp using Primer blast (NCBI) or taken from literature and used for gene expression analysis by quantitative RT-PCR. Reference genes: elongation factor 1α (eEF) and β-actin; target genes: H^+-ATPase, copper transporter 1 (CTR1), Na^+/H^+-exchanger (NHE-2) and Na^+/K^+-ATPase and metallothionein (MT).

Gene	Accession number	Primer 5'→ 3'	Annealing temperature (°C)	% GC	% efficiency
eEF	Sinha et al. (2012) AF485331.1	F - TGGAGATGCTGCCATTGT R - TGCAGACTTCGTGACCTT	58	50 50	92
β-actin	Wu et al. (2014) M24113	F - CGTGATGGACTCTGGTGATG R - TCGGCTGTGGTGGTGAAG	62	55 61.1	96
H^+-ATPase	Sinha et al. 2016 JX570880	F - CTATGGGGGTCAACATGGAG R - CCAACACGTGCTTCTCACAC	59	55 55	103
Na^+/K^+-ATPase	Castaldo et al. 2020 JX570881.1	F - ATGGGTCGTATCGCCACTCT R - CCAGGAAGACAGCAACACCA	55	55 55	104
NHE-2	XM_019098528	F - CACACAAGCTTACGACGCAG R - TCCAGTGTGAACGAGTCTCC	57	55 55	107
CTR1	XM_019104458.1	F - TCATCAACACACCAGGAGGA R - AATAGGAACTCACGGGCGAT	60	50 50	97
MT	Reynders et al. 2006	F - CCAAGACTGGAACTTGC R - ACGTTGACCTCCTCAC	60	52.9 56.3	93

SI-Table 4: Cu content, net accumulation, percentage of increase and accumulation rate, in fish gills exposed to a metal mixture for 27 days either at 10 °C or 20 °C.

Copper										
	Day 1		**Day 7**		**Day 14**		**Day 21**		**Day 27**	
10 °C	Control 10 °C	Treatment 10 °C	Control 10 °C	Treatment 10 °C	Control 10 °C	Treatment 10 °C	Control 10 °C	Treatment 10 °C	Control 10 °C	Treatment 10 ºC°C
Total metal content (nmol/g dw)	60.4 ± 3.67	96.19 ± 24.45	58.62 ± 2.19	125.58 ± 20.8	59.37 ± 4.68	123.04 ± 16.44	60.65 ± 2.32	96.19 ± 7	61.13 ± 10.99	101.48 ± 15.91
Net acc (nmol/g dw)	/	35.79 ± 24.45	/	66.97 ± 20.8	/	63.67 ± 16.44	/	35.54 ± 7	/	40.35 ± 15.91
% increase	/	59.24 ± 40.48	/	114.25 ± 35.49	/	107.24 ± 27.69	/	58.6 ± 11.54	/	66.01 ± 26.03
Accumulation rate (nmol g^{-1} dw h^{-1})	/	1.49 ± 1.02	/	0.4 ± 0.12	/	0.19 ± 0.05	/	0.07 ± 0.01	/	0.06 ± 0.02
	Day 1		**Day 7**		**Day 14**		**Day 21**		**Day 27**	
20 °C	Control 20 °C	Treatment 20 °C	Control 20 °C	Treatment 20 °C	Control 20 °C	Treatment 20 °C	Control 20 °C	Treatment 20 °C	Control 20 °C	Treatment 20 °C
Total metal content (nmol/g dw)	58.37 ± 3.57	96.81 ± 13.61	56.34 ± 4.09	96.85 ± 21.13	55.05 ± 2.5	79.99 ± 14.62	49.05 ± 4.39	68.19 ± 11.9	49.27 ± 3.25	63.44 ± 6.82
Net acc (nmol/g dw)	/	38.44 ± 13.61	/	40.51 ± 21.13	/	24.94 ± 14.62	/	19.15 ± 11.9	/	14.16 ± 6.82
% increase	/	65.85 ± 23.32	/	71.91 ± 37.51	/	45.29 ± 26.56	/	39.04 ± 24.26	/	28.74 ± 13.85
Accumulation rate (nmol g^{-1} dw h^{-1})	/	1.6 ± 0.57	/	0.24 ± 0.13	/	0.07 ± 0.04	/	0.04 ± 0.02	/	0.02 ± 0.01

SI-Table 5: Zn content, net accumulation, percentage of increase and accumulation rate, in fish gills exposed to a metal mixture for 27 days either at 10 ºC or 20 ºC.

Zinc										
	Day 1		**Day 7**		**Day 14**		**Day 21**		**Day 27**	
10 °C	Control 10 °C	Treatment 10 °C	Control 10 °C	Treatment 10 °C	Control 10 °C	Treatment 10 °C	Control 10 °C	Treatment 10 °C	Control 10 °C	Treatment 10 °C
Total metal content (µmol/g dw)	9.65 ± 1.6	8.34 ± 2.14	8.58 ± 1.97	8.16 ± 2.18	8.1 ± 2.12	8.89 ± 1.39	8.29 ± 0.81	8.15 ± 2.09	8.2 ± 1.06	9.98 ± 1.7
Net acc (µmol/g dw)	/	-1.31 ± 2.14	/	-0.42 ± 2.18	/	0.79 ± 1.39	/	-0.14 ± 2.09	/	1.78 ± 1.7
% increase	/	-13.59 ± 22.16	/	-4.87 ± 25.36	/	9.76 ± 17.1	/	-1.72 ± 25.17	/	21.66 ± 20.72
Accumulation rate (µmol g^{-1} dw h^{-1})	/	-0.055 ± 0.09	/	-0.002 ± 0.01	/	0.002 ± 0.004	/	-0.00028 ± 0.004	/	0.003 ± 0.003
	Day 1		**Day 7**		**Day 14**		**Day 21**		**Day 27**	
20 °C	Control 20 °C	Treatment 20 °C	Control 20 °C	Treatment 20 °C	Control 20 °C	Treatment 20 °C	Control 20 °C	Treatment 20 °C	Control 20 °C	Treatment 20 °C
Total metal content (µmol/g dw)	8.64 ± 0.85	8.75 ± 2.03	10.92 ± 0.56	10.34 ± 1.35	9.35 ± 2.15	10.46 ± 2.09	10.52 ± 2.13	12.39 ± 1.76	9.99 ± 1.98	13.97 ± 1.89
Net acc (µmol/g dw)	/	0.11 ± 2.03	/	-0.58 ± 1.35	/	1.11 ± 2.09	/	1.86 ± 1.76	/	3.97 ± 1.89
% increase	/	1.33 ± 23.5	/	-5.32 ± 12.34	/	11.88 ± 22.31	/	17.72 ± 16.7	/	39.78 ± 18.93
Accumulation rate (µmol g^{-1} dw h^{-1})	/	0.005 ± 0.08	/	-0.003 ± 0.008	/	0.003 ± 0.006	/	0.004 ± 0.003	/	0.006 ± 0.003

SI-Table 6: Cd content, net accumulation, percentage of increase and accumulation rate, in fish gills exposed to a metal mixture for 27 days either at 10 ºC or 20 ºC.

Cadmium										
	Day 1		**Day 7**		**Day 14**		**Day 21**		**Day 27**	
10 °C	Control 10 °C	Treatment 10 °C	Control 10 °C	Treatment 10 °C	Control 10 °C	Treatment 10 °C	Control 10 °C	Treatment 10 °C	Control 10 °C	Treatment 10 °C
Total metal content (nmol/g dw)	0.36 ± 0.04	4.46 ± 0.85	0.29 ± 0.06	29.32 ± 4.09	0.31 ± 0.08	48.76 ± 9.98	0.44 ± 0.15	70.87 ± 18.02	0.24 ± 0.12	105.39 ± 17.68
Net acc (nmol/g dw)	/	4.09 ± 0.85	/	29.03 ± 4.09	/	48.44 ± 9.98	/	70.43 ± 18.02	/	105.15 ± 17.68
% increase	/	1127.13 ± 232.98	/	10132.13 ± 1426.4	/	15566.51 ± 3207.69	/	16110.82 ± 4121.01	/	43702.71 ± 7349.01
Accumulation rate (nmol g^{-1} dw h^{-1})	/	0.17 ± 0.04	/	0.17 ± 0.02	/	0.14 ± 0.03	/	0.14 ± 0.04	/	0.16 ± 0.03
	Day 1		**Day 7**		**Day 14**		**Day 21**		**Day 27**	
20 °C	Control 20 °C	Treatment 20 °C	Control 20 °C	Treatment 20 °C	Control 20 °C	Treatment 20 °C	Control 20 °C	Treatment 20 °C	Control 20 °C	Treatment 20 °C
Total metal content (nmol/g dw)	1.23 ± 0.07	13.41 ± 5.49	0.84 ± 0.23	81 ± 12.73	0.28 ± 0.04	153.1 ± 33.24	0.38 ± 0.16	211.2 ± 32.43	0.22 ± 0.07	229.76 ± 22.85
Net acc (nmol/g dw)	/	12.18 ± 5.49	/	80.16 ± 12.73	/	152.82 ± 33.24	/	210.82 ± 32.43	/	229.53 ± 22.85
% increase	/	991.11 ± 446.69	/	9549.87 ± 1516.87	/	53814.62 ± 11706.42	/	55300.74 ± 8507.77	/	102685.13 ± 10221.85
Accumulation rate (nmol g^{-1} dw h^{-1})	/	0.51 ± 0.23	/	0.48 ± 0.08	/	0.45 ± 0.09	/	0.42 ± 0.06	/	0.35 ± 0.04

SI-Table 7: Copper, Cd (nmol/g dw), Zn and electrolyte levels (µmol/g dw) in Cyprinus carpio gills, exposed to a metal mixture for 27 days. Mean ± SD, N=5, letters indicate significant differences ($p < 0.05$). Asterisks (*) indicate differences between control and metal treatment at each sampling day, the hash (#) indicates differences between the same groups (both control and metal treated) at different temperatures and lowercase letters indicate significant differences of the same group among sampling days at each temperature ($p < 0.05$).

10 °C		**Day 1**		**Day 7**		**Day 14**		**Day 21**		**Day 27**	
		Control	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**
Gills	**Cu**	60.4 ± 3.67 a	96.19 ± 24.45 a*	58.62 ± 2.19 a	125.58 ± 20.8 b*#	59.37 ± 4.68 a	123.04 ± 16.44 ab*#	60.65 ± 2.32 a	96.19 ± 7 a*#	61.13 ± 10.99 a	101.48 ± 15.91 ab*#
	Zn	9.65 ± 1.6 A	8.34 ± 2.14 a	8.58 ± 1.97 a	8.16 ± 2.18 a	8.1 ± 2.12 a	8.89 ± 1.39 a	8.29 ± 0.81 a	8.15 ± 2.09 a#	8.2 ± 1.06 a	9.98 ± 1.7 a
	Cd	0.36 ± 0.04 ab#	4.46 ± 0.85 a*#	0.29 ± 0.06 ab#	29.32 ± 4.09 b*#	0.31 ± 0.08 ab#	48.76 ± 9.98 bc*#	0.44 ± 0.15 a	70.87 ± 18.02 cd*#	0.24 ± 0.12 b	105.39 ± 17.68 d*#
	Ca	520.55 ± 112.99	435.11 ± 68.94	467.99 ± 73.66	517.93 ± 78.04	502.93 ± 40.44	469.28 ± 53.92	489.09 ± 72.32	445.4 ± 31.1	519.06 ± 84.72	480.36 ± 46.63
	K	275.45 ± 15.17 a	272.78 ± 16.29 ab	258.76 ± 13.06 a	286.29 ± 23.27 a	268.63 ± 15.13 a	260.31 ± 27.66 ab	254.54 ± 6.85 a	228.87 ± 21.66 b	263.95 ± 38.01 a	261.97 ± 15.58 ab
	Mg	48.82 ± 3.89	43.26 ± 7.64	45.66 ± 2.69	48.41 ± 3.42	47.84 ± 2.53	46.85 ± 5.27	46.13 ± 2.33	43.08 ± 3.75	51.4 ± 4.23	48.07 ± 5.13
	Na	273.93 ± 13.5 #	252.02 ± 51.48 #	267.48 ± 15.02 #	289.44 ± 3.92	272.2 ± 10.35	254.8 ± 32.95	269.92 ± 15.08	235.97 ± 22.78	270.7 ± 41.3	269.07 ± 17.93
20 °C											
Gills	**Cu**	58.37 ± 3.57 a	96.81 ± 13.61 a*	56.34 ± 4.09 a	96.85 ± 21.13 a*	55.05 ± 2.5 a	79.99 ± 14.62 ab	49.05 ± 4.39 a	68.19 ± 11.9 b	49.27 ± 3.25 a	63.44 ± 6.82 b*
	Zn	8.64 ± 0.85 a	8.75 ± 2 .03 a	10.92 ± 0.56 a	10.34 ± 1.35 ab	9.35 ± 2.15 a	10.46 ± 2.09 ab	10.52 ± 2.13 a	12.39 ± 1.76 ab	9.99 ± 1.98 a	13.97 ± 1.89 b
	Cd	1.23 ± 0.07 a	13.41 ± 5.49 a*	0.84 ± 0.23 a	81 ± 12.73 b*	0.28 ± 0.04 b	153.1 ± 33.24 c*	0.38 ± 0.16 b	211.2 ± 3 2.43 c*	0.22 ± 0.07 b	229.76 ± 22.85 c*
	Ca	582.71 ± 75.13	594.55 ± 117.5	599.22 ± 86.83	529.09 ± 131.76	562.74 ± 86.25	509.19 ± 45.33	505.06 ± 54.15	465.49 ± 18	516.54 ± 70.33	482.83 ± 67.65
	K	271.97 ± 12.25 a	253.51 ± 24.55 a	264.69 ± 11.28 a	256.44 ± 41.06 a	267.42 ± 8.11 a	263.13 ± 18.27 a	254.84 ± 25 a	258.22 ± 11.81 a	254.1 ± 9.45 a	287 ± 8.76 a
	Mg	48.8 ± 2.92	48.2 ± 6.7	51.65 ± 3.83	45.56 ± 8.35	49.56 ± 3.89	47.75 ± 3.08	44.67 ± 3.71	43.8 ± 1.74	48.51 ± 2.64	48.69 ± 3.79
	Na	352.72 ± 21.54	322.28 ± 28.2	333.89 ± 28.32	319.97 ± 45.84	319.04 ± 21.39	297.83 ± 15.55	298.28 ± 14.28	286.74 ± 18.61	318.26 ± 12.28	320.06 ± 3.37

.

SI-Table 8: Copper, Cd (nmol/g dw), Zn and electrolyte levels (µmol/g dw) in *Cyprinus carpio* liver, exposed to a metal mixture for 27 days. Mean ± SD, N=5, letters indicate significant differences ($p < 0.05$). Asterisks (*) indicate differences between control and metal treatment at each sampling day, the hash (#) indicates differences between the same groups (both control and metal treated) at different temperatures and lowercase letters indicate significant differences of the same group among sampling days at each temperature ($p < 0.05$).

10 °C		**Day 1**		**Day 7**		**Day 14**		**Day 21**		**Day 27**	
		Control	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**
Liver	**Cu**	241.52 ± 56.57a#	210.2 ± 55.32a#	251.48 ± 38.07a#	288.84 ± 52.09a#	288.19 ± 39.04a	382.2 ± 83.42a#	278.87 ± 48.94a	333.42 ± 44.93a#	322.3 ± 73.07a	408.84 ± 51.02a
	Zn	1.02 ± 0.15a#	0.95 ± 0.07a#	1.12 ± 0.17a#	1.24 ± 0.26a	1.26 ± 0.5a	1 ± 0.09a#	1.23 ± 0.37a	1.31 ± 0.49a#	1.13 ± 0.15a#	1.52 ± 0.29a#
	Cd	0.186 ± 0.041a#	0.192 ± 0.035a#	0.151 ± 0.004a#	0.775 ± 0.724a#	0.585 ± 0.402a#	1.754 ± 0.263b#	0.708 ± 0.15a#	1.644 ± 0.122b#	0.736 ± 0.06a#	2.561 ± 0.33b#
	Ca	3.81 ± 1.56	4.15 ± 2.76	2.98 ± 1.57	2.64 ± 0.49	2.52 ± 0.76	3.6 ± 2.87	3.1 ± 1.35	3.19 ± 0.89	3.6 ± 1.29	2.39 ± 0.31
	K	183.72 ± 8.17#	182.08 ± 16.54	196.92 ± 6.14#	184.7 ± 11.32	198.52 ± 15.83	177.89 ± 22.83#	195.76 ± 10.49	199.66 ± 9.38	195.83 ± 11.29	206.33 ± 4.14
	Mg	21.8 ± 1.51#	24.25 ± 1.93#	22.95 ± 1.11#	22.63 ± 1.68#	23.62 ± 1.49#	23.19 ± 1.61#	22.96 ± 1.55#	24.03 ± 1.43#	22.77 ± 1.14#	24.9 ± 1.34#
	Na	55.84 ± 4.16#	66.18 ± 3.25#	61.82 ± 3.33#	60.65 ± 5.47#	64.09 ± 9.99#	58.5 ± 7.44#	57.73 ± 7.57#	59.62 ± 4.48#	56.94 ± 4.41#	61.07 ± 2.98#
20 °C											
Liver	**Cu**	592.11 ± 213.79a	625.83 ± 122.15a	542.81 ± 136.06ab	541.13 ± 146.52a	346.61 ± 46.86b	674.96 ± 154.56a*	490.6 ± 96.27ab	610.33 ± 114.11a	529.27 ± 92.17ab	640.53 ± 155.59a
	Zn	2.29 ± 0.32a	2.7 ± 0.92ab	2.5 ± 0.74a	2.03 ± 0.37a	2.01 ± 0.15a	2.72 ± 1.02ab	2.25 ± 0.33a	2.61 ± 0.21ab	2.36 ± 0.34a	3.48 ± 0.43b
	Cd	3.436 ± 0.5588a	5.287 ± 1.1671a	2.972 ± 0.7958ab	5.867 ± 0.3411a*	2.539 ± 0.7559ab	18.435 ± 2.7902b*	2.514 ± 0.3299ab	28.871 ± 1.6023c*	1.839 ± 0.4076b	52.065 ± 6.4067d*
	Ca	6.4 ± 3.8	5.04 ± 2.72	5.31 ± 2.61	3.54 ± 0.35	3.56 ± 0.64	4.23 ± 0.84	3.82 ± 0.23	3.9 ± 0.2	3.37 ± 0.25	4.1 ± 0.39
	K	229.76 ± 14.68	200.98 ± 15.95	244.03 ± 56.57	213.66 ± 7.57	203.21 ± 30.41	230.23 ± 19.5	223.73 ± 18	227.84 ± 9.79	218.9 ± 7.81	237.01 ± 6.67
	Mg	33.52 ± 2.25	30.66 ± 1.73	29.37 ± 4.58	32.1 ± 2.28	29.81 ± 4.51	35.14 ± 2.94*	32.74 ± 2.9	32.93 ± 1.25	31.69 ± 0.92	35.16 ± 1.66
	Na	136.97 ± 14.9a	120.52 ± 14.67a	111.31 ± 24.7b	111.3 ± 9.74a	92.07 ± 7.43b	118.74 ± 14.56a	112.88 ± 8.34b	101.56 ± 3.37a	95.1 ± 8.71b	122.56 ± 8.14a*

SI-Table 9: Metal and electrolyte levels multiplied by liver dw and expressed as nmol (Cu and Cd) or µmol (Zn and electrolytes) per total liver in *Cyprinus carpio*, exposed to a metal mixture for 27 days. Mean ± SD, N=5. Asterisks (*) indicate differences between control and metal treatment at each sampling day, the hash (#) indicates differences between the same groups (both control and metal treated) at different temperatures and lowercase letters indicate significant differences of the same group among sampling days at each temperature ($p < 0.05$).

10 °C		**Day 1**		**Day 7**		**Day 14**		**Day 21**		**Day 27**	
		Control	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**
Tot Liver (dw)	**Cu**	10.97 ± 3.85a	7.38 ± 2.61a	19.02 ± 5.24a	17.08 ± 2.73ab	16.88 ± 5.33a	20.68 ± 11.16ab	23.18 ± 12.61ab	26.8 ± 10.56bc	33.93 ± 6.68bc#	36.08 ± 8.72c#
	Zn	0.05 ± 0.02a	0.03 ± 0.01a	0.08 ± 0.01ab	0.07 ± 0.02ab	0.08 ± 0.05ab	0.05 ± 0.03a	0.11 ± 0.07ab	0.11 ± 0.07b	0.12 ± 0.02b	0.14 ± 0.04b
	Cd	0.008 ± 0.003a#	0.006 ± 0.001a#	0.011 ± 0.002ab#	0.041 ± 0.035b*#	0.031 ± 0.019bc	0.095 ± 0.046c*#	0.059 ± 0.032cd	0.13 ± 0.047cd#	0.077 ± 0.009d	0.234 ± 0.087d*#
	Ca	0.18 ± 0.1	0.15 ± 0.12	0.22 ± 0.1	0.16 ± 0.02	0.15 ± 0.07	0.16 ± 0.11	0.28 ± 0.25	0.25 ± 0.09	0.38 ± 0.14#	0.21 ± 0.06
	K	8.2 ± 1.72a#	6.39 ± 1.77a	14.85 ± 3.26ab#	11.29 ± 3.4abc#	11.45 ± 2.23ab#	9.44 ± 4.1ab#	16.08 ± 7.68ab#	15.74 ± 5.17bc#	20.84 ± 2.83b#	18.68 ± 6.19c#
	Mg	0.97 ± 0.18a	0.85 ± 0.22a	1.73 ± 0.35ab#	1.38 ± 0.41ab#	1.36 ± 0.25ab#	1.23 ± 0.51a	1.9 ± 0.95ab#	1.89 ± 0.61b#	2.42 ± 0.33b#	2.28 ± 0.84b#
	Na	2.49 ± 0.56a	2.29 ± 0.48a	4.64 ± 0.88b	3.66 ± 0.85abc	3.68 ± 0.68ab	3.05 ± 1.09ab	4.72 ± 2.21b	4.7 ± 1.52bc#	6.06 ± 0.84b#	5.58 ± 2.02c
20 °C											
Tot Liver (dw)	**Cu**	9.23 ± 2.02a	10.62 ± 2.79a	12.78 ± 3.51a	12.16 ± 3.66a	8.54 ± 1.75a	12.69 ± 4.01a	13.84 ± 4.55a	14.65 ± 2.62a	17.7 ± 6.08a	17.87 ± 5.7a
	Zn	0.04 ± 0.01a	0.04 ± 0.01a	0.06 ± 0.02a	0.05 ± 0.01a	0.05 ± 0.01a	0.05 ± 0.02a	0.06 ± 0.02a	0.06 ± 0a	0.08 ± 0.02a	0.1 ± 0.03a
	Cd	0.05 ± 0.008a	0.089 ± 0.02a	0.07 ± 0.021a	0.132 ± 0.025a	0.061 ± 0.011a	0.343 ± 0.086b*	0.069 ± 0.015a	0.696 ± 0.068bc*	0.061 ± 0.02a	1.5 ± 0.5c*
	Ca	0.11 ± 0.06	0.06 ± 0.01	0.13 ± 0.08	0.08 ± 0.01	0.09 ± 0	0.08 ± 0.02	0.11 ± 0.02	0.09 ± 0.01	0.11 ± 0.03	0.12 ± 0.03
	K	3.76 ± 0.96a	3.39 ± 0.49a	5.21 ± 0.81ab	4.85 ± 1.13ab	5 ± 0.98ab	4.28 ± 1.05ab	6.17 ± 1.16ab	5.5 ± 0.52ab	7.31 ± 2.03b	6.81 ± 1.97b
	Mg	0.55 ± 0.13a	0.52 ± 0.08a	0.76 ± 0.09ab	0.72 ± 0.13ab	0.73 ± 0.14ab	0.65 ± 0.16ab	0.9 ± 0.16ab	0.79 ± 0.07ab	1.05 ± 0.27b	1 ± 0.27b
	Na	2.21 ± 0.47 a	2.03 ± 0.31 a	2.61 ± 0.48 a	2.5 ± 0.41 a	2.27 ± 0.35 a	2.22 ± 0.61 a	3.11 ± 0.56 a	2.45 ± 0.2 a	3.17 ± 0.88 a	3.49 ± 0.89 a

SI-Table 10: Copper, Cd (nmol/g dw), Zn and electrolyte levels (µmol/g dw) in *Cyprinus carpio* brain, exposed to a metal mixture for 27 days. Mean ± SD, N=5, letters indicate significant differences ($p < 0.05$). Asterisks (*) indicate differences between control and metal treatment at each sampling day, the hash (#) indicates differences between the same groups (both control and metal treated) at different temperatures and lowercase letters indicate significant differences of the same group among sampling days at each temperature ($p < 0.05$).

10 °C		**Day 1**		**Day 7**		**Day 14**		**Day 21**		**Day 27**	
		Control	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**
Brain	**Cu**	104.14 ± 14.14	107.6 ± 4.51	103.11 ± 7.33	107.77 ± 5.55	92.66 ± 10.39	96.67 ± 4.29	95.07 ± 6.59	99.13 ± 11.8	96.57 ± 8.21	98.59 ± 10.48
	Zn	1.15 ± 0.22	1.34 ± 0.41	1.16 ± 0.09	1.13 ± 0.06	1.06 ± 0.14	1.2 ± 0.18	1.16 ± 0.1	1.15 ± 0.09	1.12 ± 0.12	1.27 ± 0.22
	Cd	BMQL	BMQL	BMQL	0.44	BMQL	1.45 ± 0.39	BMQL	1.05 ± 0.65	BMQL	0.84
	Ca	23.12 ± 14.49	146.88 ± 127.58	15 ± 6.94	119.73 ± 119.8	18.7 ± 14.23	41.47 ± 39.83	28.65 ± 23.89	43.93 ± 54.17	10.45 ± 0.79	27.12 ± 29.39
	K	338.77 ± 42.82	362.76 ± 27.66	339.57 ± 14.28	341.7 ± 6.37	336.82 ± 10.7	326.52 ± 11.74	332.02 ± 14.8 #	325.89 ± 26.5	327.44 ± 9.8	330.77 ± 5.55
	Mg	31.46 ± 3.83	34.08 ± 2.84	31.76 ± 1.26	32.98 ± 0.61	31.79 ± 0.88	31.99 ± 1.22	30.87 ± 1.1	30.28 ± 2.47	30 ± 0.99	30.9 ± 0.71
	Na	193.16 ± 29.81#	194.44 ± 4.88#	192.18 ± 11.36#	198.04 ± 10.24#	191.66 ± 5.82#	183.28 ± 8.9#	195.42 ± 6.57#	186.81 ± 14.6#	189.04 ± 8.69	190.53 ± 6.16
20 °C											
Brain	**Cu**	88.11 ± 17.26	105.1 ± 9.94	94.33 ± 8.81	96.49 ± 12.19	96.25 ± 4.98	99 ± 11.19	99.24 ± 17.49	100.2 ± 2.42	89.3 ± 6.09	94.12 ± 14.91
	Zn	0.96 ± 0.14	1.04 ± 0.1	1.03 ± 0.03	1.18 ± 0.13	1.08 ± 0.06	1.11 ± 0.09	1 ± 0.03	1.23 ± 0.05	1.03 ± 0.04	1.14 ± 0.23
	Cd	1.86	3.09	BMQL	1.16	BMQL	0.9 ± 0.32	BMQL	2.01 ± 1.64	BMQL	1.05 ± 0.19
	Ca	65.52 ± 74.85	117.64 ± 129.1	31.42 ± 21.78	140.96 ± 177.67	17.1 ± 7.5	15.83 ± 3.14	16.55 ± 6.61	37.37 ± 25.32	12.1 ± 2.41	18.67 ± 9.29
	K	341.28 ± 30.1	350.8 ± 18.19	363.77 ± 18.54	355.67 ± 15.92	346.7 ± 14.46	361.24 ± 14.44	347.71 ± 17.63	376.42 ± 12	348.95 ± 8.32	363.76 ± 3.46
	Mg	29.79 ± 3.18	30.74 ± 1.18	30.6 ± 1	30.28 ± 1.81	30.03 ± 1.09	30.28 ± 1.33	30.06 ± 1.73	32.82 ± 1.35	30.64 ± 0.86	30.95 ± 0.63
	Na	234.2 ± 7.04	237.84 ± 29.63	243.27 ± 18.42	242.1 ± 16.47	235.25 ± 18.35	248.41 ± 20.03	234.1 ± 14.59	253.95 ± 21.82	221.93 ± 7.68	222.69 ± 9.49

SI-Table 11: Copper, Cd (nmol/g dw), Zn and electrolyte levels (µmol/g dw) in *Cyprinus carpio* muscle, exposed to a metal mixture for 27 days. Mean ± SD, N=5, letters indicate significant differences ($p < 0.05$). Asterisks (*) indicate differences between control and metal treatment at each sampling day, the hash (#) indicates differences between the same groups (both control and metal treated) at different temperatures and lowercase letters indicate significant differences of the same group among sampling days at each temperature ($p < 0.05$).

10 °C		**Day 1**		**Day 7**		**Day 14**		**Day 21**		**Day 27**	
		Control	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**
Muscle	**Cu**	46.1 ± 2.03a	39.72 ± 5.99a	48.11 ± 6.94a	53.31 ± 5.97ab	50.8 ± 4.2a	56.25 ± 8.83ab	51.66 ± 7.35a	65.84 ± 13.14b	62.39 ± 7.11 a#	68.54 ± 9b#
	Zn	0.78 ± 0.21#	0.55 ± 0.15	0.54 ± 0.04	0.59 ± 0.16	0.68 ± 0.06	0.65 ± 0.24	0.62 ± 0.13	0.61 ± 0.12	0.65 ± 0.12	0.73 ± 0.09
	Cd	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL	BMQL
	Ca	104.38 ± 59.2a#	47.86 ± 17.07a	29.11 ± 1.77b	29.2 ± 5.09a	49.32 ± 16.46ab	44.46 ± 16.74a	43.6 ± 12.98b	34.94 ± 8.27a	33.02 ± 8.53 b	32.77 ± 4.36a
	K	385.44 ± 21.84	362.89 ± 57.76	372.85 ± 18.96	394.11 ± 18.84	357.85 ± 29.09	346.99 ± 29.71	337.63 ± 32.19	336.91 ± 11.95	332.56 ± 42.1	348.01 ± 28.38
	Mg	60.89 ± 3.07	56.4 ± 8.02	57.75 ± 5.32	61.14 ± 5.72	57.66 ± 7.84	57.57 ± 1.97	61.07 ± 5.06	58.09 ± 2.45	58.17 ± 8.83	58.01 ± 7.95
	Na	91.1 ± 7.53	72.84 ± 16.77	81.95 ± 9.6	84.01 ± 7.3	85.14 ± 4.52	68.2 ± 8.99	82.41 ± 7.23	81.23 ± 10.46	83.26 ± 13.14	84.02 ± 13.86
20 °C											
Muscle	**Cu**	54.89 ± 18.63a	45.9 ± 15.7a	50.63 ± 16.97a	58.2 ± 6.69a	48.95 ± 9.7a	53.43 ± 7.27a	39.97 ± 10.93a	54.51 ± 10.91a	37.38 ± 18.17a	32.62 ± 4.96a
	Zn	0.44 ± 0.12	0.46 ± 0.08	0.5 ± 0.15	0.61 ± 0.16	0.53 ± 0.11	0.64 ± 0.12	0.43 ± 0.1	0.61 ± 0.07	0.41 ± 0.1	0.54 ± 0.03
	Cd	BMQL	BMQL	BMQL	BMQL	BMQL	0.88 ± 0.11	BMQL	1.09 ± 0.62	BMQL	0.61 ± 0.23
	Ca	41.54 ± 20.38	33.89 ± 11.59	33.12 ± 11.2	37.61 ± 10.31	40.57 ± 11.73	48.75 ± 1 6.8	27.98 ± 10.43	40.56 ± 19.88	33.74 ± 10.48	30.53 ± 8.85
	K	376.72 ± 61.78	388.85 ± 81.75	367.99 ± 45.99	437.45 ± 23.85	366.32 ± 36.97	399.92 ± 25.72	339.15 ± 38.3	387.79 ± 6.04	343.28 ± 79.87	380.23 ± 41.02
	Mg	60.61 ± 10.37	62.59 ± 11.01	61.71 ± 10.42	71.4 ± 5.06	61.16 ± 7.28	65.73 ± 1.76	57.53 ± 6.59	66.21 ± 2.28	59.4 ± 15.21	63.95 ± 6.06
	Na	86.58 ± 26.83	80.6 ± 20.6	75.37 ± 11.83	85.27 ± 9.88	73.57 ± 10.14	73.2 ± 5.79	67.57 ± 7.85	79.64 ± 5.16	65.14 ± 15.9	69.33 ± 12.23

SI-Table 12: Copper, Cd (nmol/g dw), Zn and electrolyte levels (µmol/g dw) in *Cyprinus carpio* carcass, exposed to a metal mixture for 27 days. Mean ± SD, N=5, letters indicate significant differences ($p < 0.05$). Asterisks (*) indicate differences between control and metal treatment at each sampling day, the hash (#) indicates differences between the same groups (both control and metal treated) at different temperatures and lowercase letters indicate significant differences of the same group among sampling days at each temperature ($p < 0.05$).

10 °C		**Day 1**		**Day 7**		**Day 14**		**Day 21**		**Day 27**	
		Control	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**
Carcass	**Cu**	52.82 ± 3.55a	47.61 ± 2.29ab#	47.43 ± 4.68ab	50.43 ± 6.33a	44.64 ± 1.87abc	44.98 ± 7.33ab	38.86 ± 6.43bc	37.09 ± 2.77b	35.83 ± 2.93c	37.39 ± 7.23b
	Zn	3.43 ± 0.46	3.2 ± 0.25	3.01 ± 0.26	3.09 ± 0.14	2.95 ± 0.35	3.33 ± 0.51	3.13 ± 0.19	3.53 ± 0.98	2.92 ± 0.25	3.29 ± 0.5
	Cd	0.99 ± 0.18ab	1.21 ± 0.13a	0.91 ± 0.24a	1.89 ± 0.55ab	0.87 ± 0.26a	2.36 ± 0.6ab	0.62 ± 0.54ab	0.8 ± 0.2a#	0.25 ± 0.23B	3.26 ± 1.18b*
	Ca	771.36 ± 109.52a	848.4 ± 83.33a	690.5 ± 176.77ab	770.07 ± 113.45	686.81 ± 119.26ab	683.9 ± 183.61ab	481.27 ± 234.63ab	345.73 ± 41.94b#	295.68 ± 127.54b	531.62 ± 255.93a
	K	262.18 ± 16.7a#	279.32 ± 12.05a#	252.86 ± 13.26a#	263.22 ± 12.99ab#	252.04 ± 10.19a#	245.28 ± 26.58ab#	236.36 ± 15.31a#	226.57 ± 10.4b#	223.72 ± 5.76a#	228.05 ± 18.47b#
	Mg	57.51 ± 4.15a#	60.25 ± 2.33a#	55.26 ± 2.34a#	56.63 ± 1.8ab#	54.4 ± 2.5a#	55.06 ± 5.02ab#	52.66 ± 3.48a#	52.44 ± 2.6ab#	51.95 ± 2.29a#	50.44 ± 1.92b#
	Na	236.12 ± 25.35a#	229.71 ± 22.31a#	221.81 ± 15.38a#	222.42 ± 19.12a#	218.84 ± 12.05a#	192.18 ± 25.55a#	207.82 ± 16.59a#	195.04 ± 10.48a#	194.86 ± 7.49a#	198.35 ± 23.74a#
20 °C											
Carcass	**Cu**	52.48 ± 5.17a	60.42 ± 8.01a	46.14 ± 2.21ab	50.7 ± 3.26ab	48.01 ± 2.08ab	47.37 ± 5.72b	41.16 ± 4.62ab	45.32 ± 2.37b	37.35 ± 5.04b	40.69 ± 4.26b
	Zn	3.41 ± 0.95	3.95 ± 0.58	4.01 ± 0.4	4.19 ± 0.41	3.82 ± 0.5	3.75 ± 0.52	3.37 ± 0.31	3.82 ± 0.3	3.2 ± 0.34	4.11 ± 0.32
	Cd	0.62 ± 0.2a	1.87 ± 0.63a*	0.58 ± 0.04a	3.97 ± 0.34ab*	0.7 ± 0.05a	5.92 ± 1.47b*	0.38 ± 0.21a	6.98 ± 0.86b*	0.44 ± 0.25a	4.3 ± 2.56ab*
	Ca	976.09 ± 273.62ab	1195.45 ± 85.59a	1052.5 ± 119.05a	1021.83 ± 78.29a	1035.12 ± 122.38a	957.61 ± 205.18a	670.26 ± 235.08ab	873.44 ± 79.48a	584.05 ± 304.77b	433.37 ± 130.67b
	K	323.96 ± 8.1a	326.02 ± 20.1a	308.65 ± 8.63ab	310.67 ± 9.44ab	312.27 ± 9.98a	310.49 ± 25.11ab	289.78 ± 20.12ab	298.36 ± 9.93ab	273.98 ± 17.13b	274.75 ± 23.74b
	Mg	69.79 ± 3.99a	73.92 ± 3.63a	69.11 ± 5.44a	68.39 ± 2.38ab	67.98 ± 3.21a	67.12 ± 5.84ab	62.99 ± 2.27a	63.85 ± 3.27	61.53 ± 3.5a	61.42 ± 5.05b
	Na	310.41 ± 12.58a	307.75 ± 29.18a	278.31 ± 22.25a	276.73 ± 16.06ab	281.56 ± 21.02a	270 ± 30.01ab	258.02 ± 16.58a	247.08 ± 8.38b	229.04 ± 11.22a	235.22 ± 20.79b

SI-Table 13: Metal and electrolyte levels multiplied by carcass dw and expressed as nmol (Cu and Cd) or µmol (Zn and electrolytes) per total carcass in *Cyprinus carpio*, exposed to a metal mixture for 27 days. Mean ± SD, N=5. Asterisks (*) indicate differences between control and metal treatment at each sampling day, the hash (#) indicates differences between the same groups (both control and metal treated) at different temperatures and lowercase letters indicate significant differences of the same group among sampling days at each temperature ($p < 0.05$).

10 °C		**Day 1**		**Day 7**		**Day 14**		**Day 21**		**Day 27**	
		Control	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**
Tot Carcass (dw)	**Cu**	50.13 ± 9.4a	39.77 ± 6.03a	49.02 ± 4.75a	48.01 ± 6.62ab	49.59 ± 13.32a	48.81 ± 10.92ab	54.61 ± 5.87a	58.11 ± 8.07b	65.32 ± 6.15a	58.6 ± 14.36b
	Zn	3.26 ± 0.71ab	2.66 ± 0.35a	3.12 ± 0.37a	3 ± 0.71a	3.27 ± 0.96ab	3.71 ± 1.27ab	4.51 ± 1.05ab	5.6 ± 1.99b	5.36 ± 0.88b	5.23 ± 1.52b
	Cd	0.93 ± 0.21a	1 ± 0.11a	0.92 ± 0.18a	1.74 ± 0.25abc	0.91 ± 0.2a	2.48 ± 0.36bc*#	0.75 ± 0.52ab	1.24 ± 0.29ab#	0.41 ± 0.34b*#	4.22 ± 1.31c*
	Ca	724.03 ± 103.68a	703.51 ± 79.21a#	703.11 ± 151.97a#	734.25 ± 127.42a	737.68 ± 130.81a	711.97 ± 96.24a	629.71 ± 191.33a	536.15 ± 33.9a#	516.11 ± 127.19a	745.03 ± 181.08a
	K	249.45 ± 51.62a	233.47 ± 36.44a	263.42 ± 37.58a	255.72 ± 60.7a	277.07 ± 63.36ab	267.03 ± 58.95a	339.88 ± 72.28ab	354.99 ± 46.06a	410.71 ± 57.28b	373.48 ± 138.44a
	Mg	54.88 ± 12.78a	50.4 ± 8.07a	57.84 ± 10.08a	55.39 ± 14.95a	59.96 ± 14.63a	60.23 ± 14.54a	76.54 ± 20.39ab	82.14 ± 10.49a	95.64 ± 15.83b	84.27 ± 36.68a
	Na	223.95 ± 46.84a	190.97 ± 27.18a	230.5 ± 29.88a	216.15 ± 53.13ab	240.01 ± 53.31ab	208.79 ± 47.44ab	298.85 ± 65.23ab	305.58 ± 39.76ab	357.21 ± 46.38b	320.37 ± 109.13b
20 °C											
Tot Carcass (dw)	**Cu**	40.78 ± 5.87a	50.55 ± 5.36a	47.25 ± 3.31a	49.26 ± 5.74a	44.14 ± 5.37a	46.26 ± 1.95a	50.1 ± 4.07a	47.35 ± 5.26a	54.68 ± 0.61a	61.26 ± 5.94a
	Zn	3.03 ± 0.65a	3.3 ± 0.33a	4.09 ± 0.33a	4.05 ± 0.37a	3.51 ± 0.57a	3.67 ± 0.41a	4.18 ± 0.9a	3.99 ± 0.5a	4.8 ± 1.43a	6.22 ± 0.75b
	Cd	0.54 ± 0.08a	1.55 ± 0.46a*	0.59 ± 0.04a	3.84 ± 0.32b*	0.64 ± 0.06a	5.73 ± 1.1b*	0.44 ± 0.19a	7.26 ± 0.74b*	0.58 ± 0.16a	6.08 ± 2.61b*
	Ca	858.01 ± 111.43ab	1004.2 ± 95.46a	1075.42 ± 101.76a	992.38 ± 116.43a	942.72 ± 58.39ab	928.83 ± 121.97ab	787.34 ± 150.91b	908.63 ± 68.34a	778 ± 177.26b	635.08 ± 94.57b
	K	251.76 ± 45.48a	273.54 ± 19.53a	315.82 ± 9.42ab	301.81 ± 30.17ab	287.33 ± 36.85ab	305.41 ± 31.08ab	355.14 ± 44.01ab	312.62 ± 41.37ab	409.3 ± 111.53b	419.67 ± 80.69b
	Mg	54.51 ± 8.46a	62.16 ± 5.87a	70.64 ± 4.15ab	66.44 ± 6.62a	62.52 ± 8.05ab	66.32 ± 10.1a	77.59 ± 12.14ab	66.91 ± 9.35a	92 ± 25.09b	94.02 ± 19.45a
	Na	242.95 ± 40.12a	257.74 ± 1 8.79a	284.68 ± 21.01a	269.62 ± 38.02a	257.8 ± 23.3a	265.52 ± 32.32a	316.5 ± 40.76a	258.65 ± 31.77a	343.35 ± 97.75a	360.17 ± 74.85a

Chapter 7.

General discussion and future perspectives.

Metals are a worldwide threat to the aquatic ecosystems and they have been the focus of aquatic toxicology research for decades. As already mentioned in the previous chapters, trace metals, such as Zn and Cu, play a key role in aquatic organisms metabolic processes. However, when they go outside the windows of essentiality, they might exert toxic effects (Hopkin 1989, Wood 2011). In contrast metals such as Cd and Pb which are defined as non-essential, are toxic once the no-effect concentration is exceeded (Kapustka 2004). Therefore, both essential and non-essential metals might become toxic for aquatic organisms on all trophic levels (Javed and Usmani 2019).

It is known that regulatory toxicity tests are done under optimal conditions using single metals. However, aquatic organisms are simultaneously exposed to mixtures of pollutants with different environmental stressors. In addition, pollutants, when present in a mixture can show antagonistic, synergistic or additive toxic effects. Therefore the assessment of metal mixture toxicity based on results obtained from tests with single compounds at optimal conditions has a certain degree of uncertainty. The aim of the present doctoral thesis was to evaluate the adverse effects of three different metals, namely Cu, Zn and Cd not only when present alone in the exposure media, but also when present as a mixture. Moreover, even though in our studies the waterborne concentrations for each metal are different when expressed in μM, they represent similar toxicity levels and reflect environmentally relevant concentrations. Furthermore the effects of two different temperatures (10 °C and 20 °C) on a sublethal ternary metal mixture was assessed in order to understand to which extent different temperatures can affect metal mixture toxicity in common carp. In this final chapter we briefly summarize all the main findings providing general discussion, remarks and future research perspectives.

7.1. Copper, zinc and cadmium accumulation

Metal accumulation reflects the net effect of uptake and elimination rates and represents the amount of metal which is present in the target tissue. In **Chapter 2**, three parallel short-term experiments with different concentrations of Cu, Zn and Cd were conducted on common carp. The used nominal concentrations corresponded to the 25, 50 and 100% of the 96h-LC_{50} for each metal. We found that even though the metal concentrations used represent similar toxicity levels, most of the adverse effects were noticed for Cu. Considering that all the metals, with the exception of Zn accumulated quite fast and that metal content in the tissue were relatively high it is likely that some deleterious effects due to Cd might have occurred but were not picked up by our measurements. Moreover in the present experiment we noticed a negligible mortality, which occurred only in fish exposed to Cu, even at the 100% of the 96h-LC_{50} values. This lack of mortality was related, not only to the activation of defensive mechanisms (e.g. MT induction), but also to a difference in waterborne metal exposure levels, which were slightly lower than the nominal values of the 25, 50 and 100% LC_{50}

values. In fact the shapes of the LC_{50} curves were so steep for all the three metals, that the used concentrations can be considered sublethal

In **Chapters 3** and **4** the adverse effects of several binary mixtures were assessed. Regarding the Cu/Cd mixtures our results pointed out that not only a fast bioaccumulation of the two metals occurred, but that they showed a synergistic-like effect on electrolyte loss. In fact for the first time in our experiments, we observed a decreased Ca content in common carp gills. Inhibitory effects of Cd^{2+} on fish Ca^{2+}-homeostasis are well known (Cinier et al. 1997, McGeer et al. 2000b). In addition, in zebrafish Cu^{2+}, was reported to decrease Ca^{2+} uptake (Alsop and Wood 2011). Moreover, recent studies demonstrated that in zebrafish at elevated waterborne Ca^{2+} levels, a decreased Cu accumulation in the gills occurred, inferring shared uptake routes between Cu^{2+} and Ca^{2+} (Craig et al. 2010, Alsop and Wood 2011). Therefore, one might assume that also in common carp part of the Cu^{2+} epithelial transport may be via a Ca^{2+} pathway. Next to this synergistic-like effect of the two metals on electrolyte loss, we noticed a slight antagonistic-like effect of Cu on Cd uptake: the levels of accumulated Cd in the gills were lower compared to the single exposure scenario at equitoxic concentrations. Nonetheless it is worth to mention that Cu levels in the mixture were at least three times higher than Cd concentrations at similar % of the LC_{50}. Regarding the binary mixture of Cd/Zn, opposite trends were noticed between the accumulation of these two metals. On the one hand Cd, despite the inhibitory effect caused by the presence of Zn, accumulated quite fast. On the other hand, and similar to the single exposure scenario, Zn showed a delayed accumulation. When considering the binary mixture scenario, the highest Cd concentration seems to stimulate Zn accumulation. An increased Zn accumulation was observed in the mussels exposed to a mixture of Cd and Zn (at the highest Cd levels) (Elliott et al. 1986). On the one hand, one might speculate that this is due to some damages at the gill site, but in this case we would have expected also other adverse effects (e.g. electrolyte imbalance). On the other hand this might be linked to a reduction in excretory processes and Zn transfer. Moreover, we can speculate that an increased affinity for Ca^{2+} carriers, in order to keep Ca levels under control might have occurred. Nevertheless more studies are needed to fully understand this process. The mixture Cd/Zn does not appear to have major adverse effects on electrolyte levels, probably due to the protective role of ions present in the medium-hard water towards Cd^{2+} and Zn^{2+} toxicity and the relatively short exposure period.

In **Chapters 5** and **6**, common carp were exposed to ternary mixtures at concentrations reflecting 10% of the 96h LC_{50} for each metal. During the short exposure period using fasted fish, a fast metal accumulation occurred, with the exception of Zn which remained almost stable for the whole week. Moreover lower Na levels were noticed in various tissues (e.g. gills) and in the remaining carcasses and we might relate it to the presence of Cu in the mixture. However considering that the analyzed tissues are

irrorated with extracellular fluid and blood, it is likely that the observed loss reflected what is in the plasma rather than the tissue itself. Therefore, it is advisable to measure plasma and total body electrolytes to validate this thought. In the long term exposure, using fed fish at 10 °C and 20 °C, Cu and Cd again accumulated relatively fast, whereas Zn did not. Nevertheless no ion losses were noticed, probably due to the supply via food, which masked the adverse effects of the metals on gill ion transport. Moreover, in fish exposed at 20 °C the accumulated Cu in gills in the metal exposed group started to level off after one week, reaching levels similar to the control. This suggests, that such relatively low concentrations of Cu, despite the first shock phase characterized by a fast accumulation, can be handled by the fish under these rearing conditions.

As mentioned above, generally, an increasing trend in metal accumulation was noticed. However the metal increase showed some differences between the single exposure scenario and the mixture scenarios. For instance when Cu is present in a mixture with Cd we can find an antagonistic-like effect of Cd on Cu uptake at the beginning which, by the end of the exposure seems to turn in a synergistic-like effect that stimulates the Cu uptake. In **Chapter 3** when Cu is fixed, at day 1 and 3, a reduced net accumulation of Cu in the mixture as compared to the single exposure was reported in common carp gills. However, by the end of the experiment, the Cu content increased in all the treatments. When Cd is fixed, Cu content in the group Cd_{25}/Cu_{50} showed lower values in the mixture as compared to the single exposure for the whole week. This suggest that some inhibiting effect of Cd on Cu accumulation is present but that the dose-dependency is not very clear.

On the contrary the inhibiting effect of Cu on Cd uptake seems to be well defined in the mixture leading to a reduced accumulation of at least $\simeq 4.7$ times as compared to the single scenario. Thus, the presence of shared uptake routes for the two metals (e.g. ECaC, DMT1 and Zip) and non-specific competition for binding sites might explain the observed trends. A similar observation on Cd accumulation occurred also in the mixture Cd/Zn (**Chapter 4**). However, the inhibitory effect of Zn on Cd accumulation did not last until the end of the experiment. Probably the higher binding constant of Cd and a reduced affinity of the transporters to Zn helped in this case.

Regarding Zn on the contrary, a, inhibition due to the presence of Cd in the mixture as compared to the single exposure scenario took place. Again this inhibitory effect might be related to shared uptake routes and to the ability of Cd to bind more strongly the cysteine proteins. Nonetheless, it is worth to mention that due to the high difference in waterborne metal levels between Cd and Zn in the mixture scenario, the inhibitory effects of different Cd concentrations on Zn levels were not always so marked.

When the three metals are present it appears that a synergistic-like effect of Zn and Cd on Cu uptake occurred, leading to a slightly higher Cu accumulation ($\simeq 48\ \%$) in

the mixture by the end of the experiment compared to the single scenario. In contrast, the presence of Cu led to a reduced accumulation both Zn and Cd. Therefore, it is likely that in this case a reciprocal inhibition of metal uptake occurred.

In freshwater fish gills (Fig. 1), the uptake of the three metals might occur via several specific channels, as described above, or via competition for channels involved in ion-uptake. Nevertheless, the presence of metals can directly or indirectly affect the affinity and the efficiency of these transporters. For instance, it is known that Zn homeostasis is tightly regulated (Bury et al. 2003) and that in condition of Zn depletion or supplementation, fish can modify the expression of channel involved in its uptake/extrusion such as the Zip10 and the Znt1 (Zheng et al. 2008). Also the expression of Ecac can be modulated in response to Zn deficiency or abundance, showing a transient decrease of expression during zinc excess (Zheng et al. 2014). These changes, in case of multi-metal exposure scenarios might also help in slowing down the accumulation of metals which compete with Zn^{2+} for their uptake such as Cd^{2+} or Cu^{2+}. In fact, several studies reported reduced Cu^{2+} accumulation in presence of elevated concentrations of Ca^{2+} suggesting that they might share some uptake routes (Craig et al. 2010, Alsop and Wood 2011). Furthermore the metal mixtures used in the present thesis appeared to affect also channels or transporters involved with Cu uptake, whose accumulation at a first instance seems to be reduced due to the presence of Cd^{2+}. One might think that a combined effect of the two metals led to an initial inhibition of transport mechanisms involved in several processes (e.g Na^{+} uptake). However, this might have indirectly stimulated a response, which led to an overshoot of Cu^{2+}. Considering that the presence of Cu results in a decreased amount of Na^{+} a compensatory mechanism such as an increase in the proton pump might occur. However, even though the extrusion of protons gives the electromotive force for the Na^{+} uptake, one might assume that it can indirectly facilitate the uptake of Cu^{+} via the CTR1.

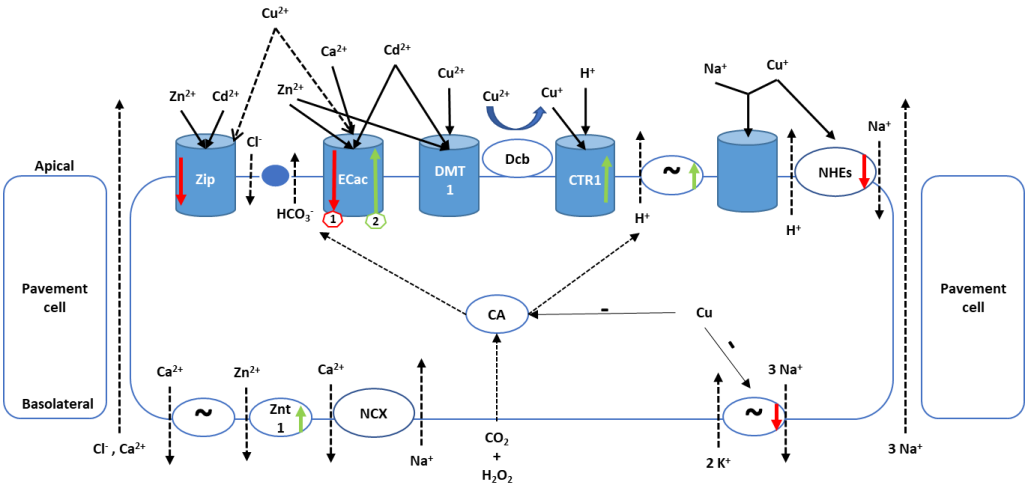


Figure 1: Schematic representation of suggested Cu, Zn and Cd uptake in freshwater fish gills using information derived from the obtained results and from the literature (Grosell 2011, Hogstrand 2011, McGeer et al. 2011, Komjarova and Bury 2014). In the figures are represented the putative apical Na^+ channel, the ATPase pumps (~), the zinc importer (e.g Zip10), the epithelial calcium channel (ECac), the divalent metal ion transporter (DMT1), the Cu reductase (Dcb), the high affinity Cu transporter (CTR1), the Na^+/Ca^{2+}-exchanger (NCX), the Na^+/H^+-exchangers (NHEs) and the carbonic anhydrase (CA). The red and the green arrows indicate respectively where a possible inhibition and stimulation occurs. For the ECac the numbers 1 and 2 indicates the sequence of events.

7.2. Ionoregulation

One of the key requirements for normal physiological processes in freshwater fish, is the maintenance of ion-homeostasis. In the present thesis, changes in the major electrolyte levels (Na, Mg, K and Ca) in tissues were assessed after metal exposure. However, it is worth to mention that in our analysis we used the whole tissue homogenate and in case of highly blood-irrorated tissues (e.g. the gills) the electrolyte measured (e.g. Na) might reflect the situation in the plasma. As discussed in **Chapter 2**, the only metal that showed to alter ion-homeostasis in common carp when present alone was Cu. Moreover the only noticeable effect occurred for Na, which already started to decrease after one day of exposure in fish exposed to the highest dose. From day 3 onwards, a Na loss was reported in all the treatments. This Na loss has been related to the ability of Cu, not only to compete with Na for the uptake sites but also with the ability of this metal to inhibit the activity of the Na^+/K^+-ATPase (De Boeck et al. 2001, Grosell 2011). In case of a Na^+/K^+-ATPase inhibition, Na ions are not efficiently eliminated from the cell and one would expect to find more Na in the cell and thus into the tissue. Therefore, it is likely that our results reflect the loss of Na content in the plasma and extracellular fluid. Nevertheless, we might speculate that a whole body loss occurred. As shown by our data, the organism was trying to cope with this situation through the activation of genes coding for the H^+-ATPase and the Na^+/K^+-ATPase. Even though the Na content in the whole body was not analysed, it is likely that the whole body sodium decrease remained below the threshold to cause massive fish mortality. For example in rainbow trout and yellow perch, mortality occurred when body Na loss reached the threshold of ~ 30 % and 40 % (Taylor et al. 2003), whereas in gibel carp the onset of the mortality is set at an electrolyte loss of ~ 45 % (De Boeck et al. 2010b).

When common carp was exposed to a mixture of metals, such as the binary Cu/Cd (**Chapter 3**) and Cd/Zn (**Chapter 4**), the effects of metal on electrolyte levels were slightly different according to the mixture. For instance in the Cu/Cd series a strong effect of the mixtures on Na levels in the gills together with a more gentle effect on Ca was observed. On the other hand no substantial differences were observed in electrolyte content in the Cd/Zn mixture. These findings suggested that synergistic-like effects occurred between Cu and Cd on the activity of ion-transporters and enzymes involved in ion-homeostasis. Moreover, the mixture Cu/Cd was the only experiment in which a lower Ca level in the gills was reported indicating and supporting the thought that the effects of Cu and Cd are more than additive. In the Cd/Zn mixture, even though both metals are known to be able to interact with Ca and cause hypocalcaemia (McGeer et al. 2000b, Hogstrand 2011, McGeer et al. 2011), no effects were found on Ca levels. One might assume that adjustments were made to favour Ca uptake from the water. For instance, in rainbow trout exposed to Zn there was an increase in the affinity of the Ca^{2+} carriers during the first days of exposure, which decreased from day

7 onwards, probably in order to limit Zn uptake (Hogstrand et al. 1995). Moreover, after 7 days of exposure, a restoration of the Ca^{2+} transporting capacity in order to maintain plasma Ca homeostasis even with a decreased affinity of the transporting sites, occurred (Hogstrand et al. 1995). Furthermore, in general metal toxicity decreases with increasing water hardness, due to competition between metal ions and Ca^{2+} and Mg^{2+} ions (Javid et al. 2007, Ebrahimpour et al. 2010, Kim et al. 2010), thus the lack of effects on electrolyte levels could be attributed to the protective role that ambient Ca plays towards metal toxicity (Hogstrand et al. 1994, Hollis et al. 2000b), to the relatively low waterborne metal concentrations and to the short exposure period. Nevertheless, Cu appears to be the key metal in these scenarios, exceeding the protective effects of water hardness and leading to more deleterious effects. Nonetheless it is important to consider that lower Ca levels occurred only in fish exposed to Cu_{25}/Cd_{50}. Therefore a certain threshold of metals is needed. However, it is worth to mention that in this thesis we did not specifically measure plasma Ca levels, therefore it is possible that some changes, which were not picked up by our measurements, occurred.

If we consider the case of a ternary metal exposure (**Chapter 5**) using relatively low waterborne metal levels, which correspond to the 10 % of the 96h-LC_{50}, the synergistic-like adverse effect between Cu and Cd, and in this case also Zn was confirmed for Na but not for Ca. In almost all the chapters, the organism tried to regulate and counteract the Na loss through the activation of the genes coding for enzymes and transporters involved in ion uptake (e.g. H^+-ATPase).

Even though the adverse effects of these metals, in particular Cu, on Na homeostasis were confirmed in almost all the mixtures, the results differed when the fish had access to food, as shown in **Chapter 6** and as a result, the metal-related effects on gill electrolyte transport might go unnoticed. The role of the food, as well as of temperature will be discussed in the following paragraphs.

7.3. Effects of extra variables

7.3.1. Effects of the temperature

As already mentioned, there is an emerging interest in understanding the effects of multiple stressors on aquatic ecosystems. In **Chapter 6**, the effects of two different temperatures, 10 °C and 20 °C on metal toxicity in common carp was assessed. For essential metals, our data showed the ability of common carp to handle these at 20 °C, whereas at 10 °C such ability was not so clear. The results obtained in the gills suggested that the depuration and regulation processes for essential metals over a long exposure period were more efficient at 20 °C than at 10 °C. In fact, the initially elevated amount of accumulated Cu returned to control levels at 20°C. In contrast, for the non-essential metal Cd, a continuous increase in metal levels was observed which

was more marked at 20 °C. Even though the role of temperature on organismal fitness has been pointed out (e.g. CF, HIS, liver size) (Bouchard and Guderley 2003, Vergauwen et al. 2010, Sappal et al. 2015, Pilehvar et al. 2019), in this experiment we showed how this environmental parameter might actually affect the outcome of the chemical stressor (e.g. metal bioaccumulation and depuration). Despite the strong effect of the temperature on the liver size in the cold exposure scenario, which led to higher metal levels (both in the control and in the metal-treated groups), its role on electrolyte levels was relatively marginal. In fact only limited changes occurred in electrolyte content between the two thermal scenarios, with lower values at 10 °C (mainly Na in the gills). Besides our own data shown in **Chapter 6**, the effects of a sublethal ternary mixture, using comparable metal concentrations and temperatures were assessed on the physiological performance of common carp by Pillet et al. (2021) in a one week experiment. The data obtained by Pillet et al. (2021) showed a strong impact of the temperature on common carp metabolic rates, while the effects of the metal mixture were minimal. As expected, the SMR (standard metabolic rate), defined as the minimal metabolic demand required to sustain life in fasting and resting animals (Fry 1971) was lower at 10 °C compared to 20 °C. This was in accordance with our results on Cd levels in the gills, whose accumulation was slower at the low temperature, possibly linked to lower ventilation rates due to the lower metabolic rate. Histological changes in the gills were transient and mild. Together with the presence of food, these factors might explain why almost no electrolyte level changes were observed. Furthermore, next to the increased SMR, an increased haematocrit percentage was observed in fish kept at 20 °C. Similarly to what formulated for the SMR, the authors suggested that such increase might be related to the increased ventilation and metabolic rate, where to higher release of red blood cells from the spleen would help to increase oxygen transport and maintain aerobic performance.

In the present work, the use of a relatively low feeding rate (2 % of the biomass) together with a higher metabolic demand in fish kept at the warm temperature might have contributed to the loss of condition indices. In our experiments, the results obtained for the HSI, suggests that fish exposed at 20 °C received a smaller portion of the energy required for their (higher) metabolic demand via their food compared to fish exposed at 10 °C. This might have had implications on fish energy reserves and growth, the warm temperature might have led to reduced nutrient stores and to a reduced capacity to convert nutrients in biomass (Sappal et al. 2015).

7.3.2. Effects of feeding

Even though it is known that dietary factors might have noticeable effects on fish physiology and metabolism (Cowey and Sargent 1972), the possible modifying effects of food-related variables on the toxicity and homeostatic regulation of metals have been mostly forsaken in aquatic toxicology (Lanno et al. 1989). Nevertheless, in the

past years more attention has been given to the interaction between food and metal toxicity. For instance several studies focused on the effects of food on fish performance during waterborne metal exposure (Kamunde and Wood 2003, Kjoss et al. 2005, Niyogi et al. 2006). However, to the authors knowledge, most of the studies have been conducted on single metals, therefore there is a lack of information about the combined effects of metal mixtures and food. In **chapter 5** and **6**, common carp were exposed to a comparable metal mixture representing the 10% of the 96h-LC_{50} for each metal, but in chapter 5 fish were fasted whereas in chapter 6 fish were fed. Therefore, we merged the data obtained on fish gill metal accumulation and Na levels together in order to have a general overview of the role of food (see Fig. 2). Gills, were selected because the gill transporters are influenced by feeding. For instance when dietary Cu concentrations supplied via food are low, an elevated metal uptake by gills occurs (Kamunde et al. 2002b), similarly also Zn levels taken up by the water increase with decreased supply via food (Hogstrand 2011). In addition the gills were also selected because they represent a large exposure surface area with only a short diffusion distance between the internal and external environment, thus making them as primary target for metal contamination (Brungs et al. 1973, Buckley et al. 1982). Even though Zn levels show some variation within groups, most of the changes occurred for Cu and Cd bioaccumulation, showing opposite trends. Even though both metal levels were significantly higher in the metal-treated groups, the essential element Cu showed higher levels in fasted fish as compared to fed fish, whereas the opposite was true for Cd. Copper, being an essential element, is required for the normal functions of the organism and it can be acquired via food or via water. Under normal conditions, the majority of Cu is obtained from the diet. However, when the dietary Cu concentrations are reduced, the gills efforts raise up to more than 60% of the whole body Cu uptake (Grosell 2011). Nevertheless, in our case a such increase seems counterintuitive. However it has been suggested that gill Cu uptake pathways are somehow regulated based on organismal Cu status (Kamunde et al. 2002b), therefore one might assume that Cu from the gills is transferred via the blood stream to other tissues. The results obtained for Cu content in the carcass seems to confirm this thought. The increase reported in the carcasses by the end of the experiment suggests that the continuous metal uptake had impacted regulation processes and that the depuration processes were no longer able to cope with metal uptake. Nevertheless, more specific studies are necessary to validate these thoughts. For Cd, fasted fish accumulated less metal as compared to the fed fish. During fasting the number of gill transporters to take up essential metals and electrolytes increase, thus the Na transport mechanism to acquire enough Na might be much more upregulated than Ca uptake mechanisms, which might have led to an increased Cu but not necessarily Cd uptake. This thought seems in line with Na levels reported in the gills, in fact, fasted fish showed lower levels of this electrolyte thus is no surprise that the organisms is trying to counteract this situation. Moreover, considering that under

normal dietary circumstances, the gills provide approximately the 10% of the required Cu it is likely that the higher Cd content in fed fish is related to the higher binding constant of Cd. Regarding the electrolyte Na, even though no differences were observed between the two metal exposed groups of the two experiments, it seems that in fasted fish, the ion levels of the metal-treated group were significantly lower as compared to the control. Therefore the lack of observations between fed and starved fish might be linked to some biological variation between the two batches of fish. Nevertheless the role of the food as supplier of nutrients appears to be confirmed by the stable Na levels in the metal-treated group observed in fed fish. The authors are aware of the limitation of this comparison, but it is intended as suggestion for further studies clearly meant to observe the effects of different feeding regimes on waterborne metal mixtures.

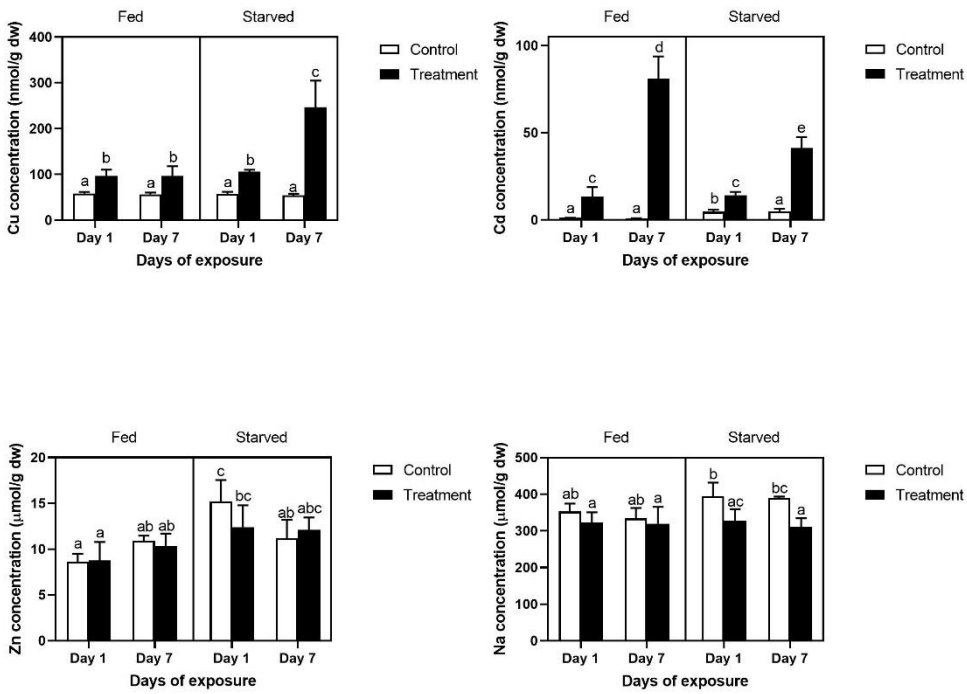


Figure 2: Copper, zinc, cadmium and sodium content in gills of *Cyprinus carpio.* The data obtained in Chapter 5 and 6 at day 1 and 7 were merged and analysed together by means of the three-way analysis of variance (ANOVA). Mean ± SD, N = 5, letters indicate significant differences ($p < 0.05$).

7.4. Defensive mechanisms

As already mentioned, metals are important contaminants of aquatic ecosystems and as a consequence of increased industrialization, metal pollution has increased and persists in water and sediments (Luoma and Rainbow 2008, Sevcikova et al. 2011). Metals, among the other toxic effects, are well known inducers of oxidative stress (Livingstone 2003), therefore fish have defensive mechanisms to cope with metal-induced toxicity. Oxidative stress is the result of an imbalanced reactive oxygen species (ROS) and antioxidant defence system (Livingstone 2001, Lushchak 2011). In the experimental chapters of this thesis we investigated the response of the organisms to metals in terms of changes in the gene expression of enzymes and proteins involved in metal sequestration and ROS depletion. In general our data showed a prompt and decisive response to waterborne metals. This is especially true for the metallothionein gene expression which, especially in the gills, was almost always increased. Even though the MT protein levels were not assessed in the previous chapters, one might assume that following the increase in gene expression, a production of the protein occurred. Moreover the discrepancy in the response observed between the gills and the liver might be linked with the background levels of the protein, which are always relatively high in the liver of common carp (Hashemi et al. 2008b). However, it is worth to mention that some authors hypothesized the presence of post-transcriptional regulation operated by stress granules (Ferro et al. 2015, Chatzidimitriou et al. 2020). The stress granules are ribonucleoprotein (RNPs) that assemble in response to environmental stresses, such as heat shock, osmotic shock or oxidative stress (Schisa 2012). It is known that the mRNAs of stress proteins can be stored in cytoplasmic foci, such as the processing bodies (P-bodies, which are cytoplasmatic granules defined as sites of mRNA storage, reversible mRNA repression and mRNA decay) or stress granules where they undergo degradation or future translation, respectively (Lavut and Raveh 2012, Olszewska et al. 2012). The stress granules can extend the half-life of stress molecules by stabilizing the mRNA contained in them, allowing a fast response to stress. Therefore next to the first hypothesis of the different background levels of the protein and taking into account the role of MT in essential elements homeostasis, one might assume that the general delay observed in the liver MT gene expression might be due to the already present mRNA levels, which were already translated into active proteins. However, it is worth to mention that different isoforms of MT are present in common carp and in the present studies we analysed the MT-1. Therefore, even though in common carp it has been demonstrated that the MT-1 is more expressed in the liver compared to the MT-2 (Hermesz et al. 2001) more studies, investigating both changes in the gene expression of MT-1 and MT-2 and in protein levels are needed to confirm this thought.

If we compare the results obtained for the MT between our experimental chapters, it seems that the increase observed in the liver for the mixture Cu_{fix}/Cd_{var} appears to

correlate better to the presence of Cu rather than Cd. In fact also in the single exposures, fish exposed to approximately Cu_{25} showed a similar trend to the one observed in the mixture. This might be linked with the fact that Cu levels in the liver are higher compared to Cd and Cu might have displaced the Zn (which is normally bound to MT), which in turn stimulated the MT response interacting with the MTF-1. If we make a similar comparison, also the increase reported in the mixture of Zn and Cd seems to be more related with the presence of Zn rather than Cd. However in the single exposure scenarios metal content in the liver was not investigated, thus it is difficult to make this kind of comparison. Moreover in order to better understand how the mixture design can influence the response of the organism, it is advisable to run the experiments in parallel using fish from the same batch.

Almost no correlation (Pearson) between metal content and MT induction in either the gills or in the liver were noticed (see Appendix tables I to VI). However in the gills a positive correlation ($P < 0.05$) can be observed in fish exposed to a mixture of Cu/Cd (e.g. Cu_{fix}/Cd_{25} and $Cd_{fix}/Cu_{10\text{-}25}$) at day 7. This suggests that the organism is responding to the metal increase. In the exposures Cd_{fix}/Zn_{10} and Cd_{fix}/Zn_{50} a negative correlation can observed between the MT and Zn and Cd levels in the tissue. This negative correlation can not be easily explained and it might be attributed to the fact that Zn homeostasis is well regulated in fish, and a small increase in free Zn (not necessarily significant) can stimulate the production of MT. In the liver a negative correlation was found between Cd levels and MT in the Cu_{fix}/Cd_{25} and Cd_{fix}/Cu_{10} experiment respectively at day 7 and day 1. However this can be linked to the fact that in the liver, the background MT protein levels were already enough to cope with the metal load and thus we observed only a transient increase in the MT gene expression. If we analyse the relationship between net accumulated metal levels in the tissue with the MT induction slightly more correlations can be noticed. For instance, in the gill tissue, a positive correlation between Zn content and MT induction at day 7 can be observed in the single exposure scenario. Similarly to what mentioned above, using net accumulated metal values, a positive correlation in the Cu/Cd mixtures can be observed. In the liver both positive and negative correlations can be noticed, however these observations are not easily explained (e.g. positive correlation without net metal accumulation in a tissue/induction of the gene coding for MT) therefore more detailed studies, taking into account the already present basal level of the MT proteins are needed. In fact, we have to keep in mind that an increase in gene expression not always reflect an increase in protein levels, thus a comparison between metal accumulation, induction of the gene coding for the MT and MT protein levels in the tissue might be more realistic and give more information.

Regarding the expression of genes coding for antioxidant enzymes, in nearly all the experiments most of the changes were noticed for the glutathione reductase (GR), which showed a slight elevation in mRNA levels in the metal-treated fish. The GR, is

known to play a role in the balance between oxidized and reduced glutathione (Dautremepuits et al. 2009). The latter has a key role as first line of defence in metal ions binding (Eyckmans et al. 2011). Thus the changes in the GR gene expression might be an attempt of the fish to reduce the oxidized glutathione, and in this way, safeguard the free radical scavenging ability of the cells. Moreover, the expression of the glutathione reductase has been demonstrated to be upregulated by Cd, showing the importance of glutathione in the cellular cadmium response (Wimmer et al. 2005).

In a study done by Pillet et al. (2019), which was run in parallel to the experiment presented in **Chapter 5**, common carp was exposed for one week to a ternary mixture of Cu, Zn and Cd at a concentration representing the 10 % of the 96h- LC_{50} for each metal. Briefly the authors investigated the adverse effects of the metal mixture on several endpoints such as oxidative stress induction, indicators of apoptosis and activity of enzymes involved in antioxidant defences. Regarding the activity of enzymes such as the GR, higher gene expression was not accompanied by increased enzymatic activity, which remained stable during the experiment. On the one hand, this might suggest a regulation at translational level (Defo et al. 2015) and on the other hand, as suggested by Pillet et al. (2019), it might be an indication of pre-adaptation mechanisms, allowing the organism to be prepared and increase the antioxidant defence when necessary. Moreover this though is in line with what was previously mentioned regarding the stress granules. Furthermore, in their experiment Pillet et al. (2019) observed no signs of oxidative stress neither in the gills or in the liver of common carp, in fact the xanthine oxidase activity (XO) and the malondialdehyde (MDA) level were almost stable for the whole experiment. This appears to be in accordance with their results on apoptosis signalling (caspase 9), which increased at the beginning of the experiment and immediately returned to control levels. In the experimental chapters of this thesis, as already mentioned, few changes in the expression of genes involved in ROS removal were reported. One might assume that similarly to what reported by Pillet et al. (2019) this might be an indication of low ROS generation rate and low oxidative stress. Therefore, MT levels might have been sufficient to protect the organism scavenging the ROS. In addition, our observations do not imply that an increased ROS generation rate is not induced by the accumulated metals. In fact in **Chapter 4**, some signs of apoptosis were observed by the end of the experiment. Therefore, one might assume that waterborne metal levels and accumulated concentrations are important factors in ROS production. Moreover, the gene expression analysis alone can only give an indication of what are the organism needs and some changes might be masked by the post-transcriptional regulation operated through stress granules. Therefore further studies assessing post-transcriptional events are needed to validate these thoughts.

7.5. Overall conclusions and future perspectives

Aquatic ecosystems are threatened by pollutant mixtures and multiple stressors. Nevertheless the classic aquatic toxicology studies on metals are still mainly limited to single exposure scenarios, using concentrations that often do not reflect environmental metal levels. Moreover in these tests, the organisms are kept under optimal conditions. Thus the potential for risk underestimation as well as overestimation arises. In fact the prediction of combined metal adverse effects has a certain degree of difficulty. Depending on the mixture some metals might accumulate more easily, leading to damages at, for instance, the gill surface and to a more accentuated ion loss. On the other hand, if inhibitory effects arise, the presence of the mixture might lead to lower levels of accumulated metal. Nevertheless even though the presence of one metal (e.g. Cu) can reduce the accumulation of another metal (e.g. Cd), they can show synergistic-like toxic effects, such as a more accentuated electrolyte imbalance, as observed in our experiments. Accordingly, our findings contribute to a better understanding of metal interaction and their adverse effects on the organism. Moreover, in this thesis, we highlighted the importance of considering the effects of an environmental parameter such as the temperature on the organismal fitness and consequently on the levels of accumulated metals and the response in terms of depuration processes. In addition side findings related to the presence of food on metal accumulation levels and ion-homeostasis were observed. Furthermore, in the present thesis the fish were able to respond adequately to the presence of metals, but some damage occurred as shown by the induction of apoptosis signalling. However, the fish were able to cope with this situation by enhancing defensive mechanisms.

Finally, in light of the present observation more tests to further expand the knowledge on these three metals are needed. For instance with the integration of the so called “omics” techniques (genomics, proteomic and metabolomics) it will be possible to study not only changes at gene levels but also the proteome and the metabolome. This will allow a better understanding of the response of the organism to stressful situations. For instance, by combining environmentally relevant concentrations and the above mentioned techniques, it will be possible to develop molecular biomarkers to predict effects on model organisms not only at physiological but also at population level in the field. Furthermore, it is advisable to compare laboratory data with data generated in more “realistic” scenarios, such as mesocosm and cage experiments (Delahaut et al. 2019). This will show how relevant and predictive this type of information is. Moreover, more studies on the interaction of metal mixtures together with natural stressors, not only limited to temperature, but including the fluctuation in salinity and pH are needed. The data should be integrated on different levels of biological organization to construct more accurate models to predict the adverse effects of multiple stressors on aquatic organisms. In addition this will allow us to determine safety margins in ecological risk assessment (Sokolova and Lannig 2008).

Furthermore it is advised to further investigate not only the role of the food on metal toxicity, but also the adverse effects of metals on fish predatory behaviour, which might alter the quantity of available food for the organism. Finally, the present thesis aimed to provide new insights into the context of multi-metal and multi-stressors exposure scenarios in common carp. However, it is clear that ecotoxicological studies and approaches are still evolving taking into account several parameters in order to predict with more accuracy metal mixtures toxicities and to improve environmental risk assessment.

Appendix

I. Extra information gene expression

Aliquots of gill and liver samples (~30 - 50 mg), were used for gene expression analysis. Total RNA was extracted according to the manufacturer protocol using Trizol (Invitrogen, Merelbeke, Belgium). In order to determine the quantity and the quality of the RNA, the Nano-Drop spectrophotometry (NanoDrop Technologies, Wilmington, DE) was used. Furthermore RNA integrity was assessed with a 1 % agarose gel with ethidium bromide (500 µg/mL). DNase treatment was performed with the commercial kit DNase I, RNase free kit from Thermo Fisher Scientific (Waltham, MA, USA). The RNA (1 µg) was transcribed to cDNA according to RevertAid H minus First strand cDNA synthesis kit protocol (Thermo fisher, Fermentas, Cambridgeshire). Four samples were selected according to the OD260/OD280 and OD260/OD230 nm absorption ratios (higher than 1.8 and 2.0 respectively) and used for qPCR. The assay was performed following the Brilliant III Ultra-Fast QPCR Master Mix (Agilent) protocol for Agilent Mx3005P QPCR system in a final reaction volume of 20 µl. The reaction mixture contained 10 µl of Brilliant III Ultra-Fast QPCR Master Mix, 5.7 µl of sterile water, 500 nM of each primer,0.3 µl of reference dye and 5 ng of cDNA. All the reactions were performed in duplicate. The contamination of reagent was assessed including the "no template" control (e.g. sterile water) in the analysis. The general experimental run protocol as described by Shrivastava et al. (2017), consisted of a denaturation program (3 min at 95°C), an amplification and quantification program repeated 40 times (15 seconds at 95 °C, 20 seconds at 60 °C) followed by a melting curve program (60 °C– 95 °C). Oligonucleotides primers were taken from literature or were designed using NCBI resources Primer blast and synthesized as highly purified salt-free "OliGold" primers by Eurogentec (Eurogentec, Seraing, Belgium). Quantification cycles (Cq) values were automatically calculated on the log curve for each gene with MxPro qPCR software (Agilent Technologies, Waldbronn, Germany). The stability of the reference genes was tested by two-ways ANOVA both in liver and in the gills. The presence of unique PCR product was assessed by means of the melting curve and the PCR product was verified on agarose gel. The primer efficiency was determined by standard curve using serial dilution of cDNA (1:10) and calculated according to the equation:

Efficiency = 10^(-1/slope) - 1.

The housekeeping genes used were the β-actin and the elongation factor 1α (EF1α) and their average was used to calculate the gene expression. Both the β-actin and the EF1α genes have been in common carp exposed to metals (Eyckmans et al. 2010, Jiang et al. 2016, Sinha et al. 2016, Zhang et al. 2017, Fazelan et al. 2020). Quantification of the target genes normalized to the reference gene and relative to the control (untreated fish) (Bustin et al. 2009) was calculated using the $2^{-\Delta\Delta Ct}$ method. Were ΔΔCt = ΔCt (treated sample) – ΔCt (untreated samples average). ΔCt = Ct (gene of interest) – Ct (housekeeping gene).

Nowadays it is recommended for qPCR analysis to test several housekeeping genes and chose the three more stable for the analysis (Vandesompele et al. 2002). Therefore we are aware of the limitation of our studies. When planning future experiments more housekeeping genes will be tested and included in the analysis.

The ratio between the housekeeping genes (β-actin/EF1α) has been calculated for each metal in the single exposure scenario. In the gill tissue the ratio is ≃ 0.95, 0.89 and 0.80, respectively for Cu, Zn and Cd, whereas in the liver tissue the ratio is ≃ 0.96, 1.03 and 1.02 for Cu, Zn and Cd respectively.

The stability of the housekeeping genes were tested by a two way ANOVA in order to observe if they are affected by the treatment.

In the liver tissue β-actin in the copper exposure: interaction F (3, 24) = 2.386 P=0.0941; duration F (1, 24) = 0.3669 P=0.5504, condition F (3, 24) = 0.6110 P=0.6144; in the zinc exposure: interaction F (3, 24) = 0.5274 P=0.6677, duration F (1, 24) = 4.029 P=0.0561, condition F (3, 24) = 0.6942 P=0.5646; in the cadmium: exposure interaction F (3, 24) = 0.7001 P=0.5612, duration F (1, 24) = 4.103 P=0.0541, condition F (3, 24) = 1.276 P=0.3051. Results for EF1α showed in the copper exposure: interaction F (3, 24) = 1.421 P=0.2611, duration F (1, 24) = 2.787 P=0.1080, condition F (3, 24) = 1.701 P=0.1935; in the zinc exposure: interaction F (3, 24) = 0.5274 P=0.6677, duration F (1, 24) = 4.029 P=0.0561, condition F (3, 24) = 0.6942 P=0.5646; in the cadmium exposure: interaction F (3, 24) = 0.7001 P=0.5612, duration F (1, 24) = 4.103 P=0.0541; condition F (3, 24) = 1.276 P=0.3051.

In the gill tissue β-actin in the copper exposure: interaction F (3, 24) = 0.3330 P=0.8016, duration F (1, 24) = 12.97 P=0.0014*, condition F (3, 24) = 2.847 P=0.0588; in the zinc exposure: interaction F (3, 20) = 0.3734 P=0.7731, duration F (1, 20) = 3.644 P=0.0707, condition F (3, 20) = 0.05221 P=0.9838; in the cadmium exposure: interaction F (3, 24) = 0.2047 P=0.8921, duration F (1, 24) = 3.410 P=0.0772, condition F (3, 24) = 0.4275 P=0.7351. Results for EF1α in the copper exposure: for interaction F (1, 12) = 3.042 P=0.1067, duration F (1, 12) = 8.506 P=0.0129*, condition F (1, 12) = 72.60 P<0.0001*; in the zinc exposure: interaction F (3, 20) = 0.3734 P=0.7731, duration F (1, 20) = 3.644 P=0.0707, F (3, 20) = 0.05221 P=0.9838; in the cadmium exposure: interaction F (3, 23) = 4.413 P=0.0136, duration F (1, 23) = 0.008704 P=0.9265, condition F (3, 23) = 2.897 P=0.0569.

The stability of the housekeeping genes was also assessed in the mixture scenarios.

In the binary mixture of Cd an Zn, β-actin gills: duration F (2, 36) = 1.698 P=0.1973; condition F (3, 36) = 1.719 P=0.1805 and their interaction F (6, 36) = 0.2158 P=0.9693; β-actin liver: duration F (2, 32) = 1.505 P=0.2373; condition F (3, 32) = 2.778 P=0.0571 and their interaction F (6, 32) = 1.238 P=0.3132; EF1α gills: duration F (2, 36) = 0.03979

P=0.9610; condition F (3, 36) = 1.682 P=0.1882 and their interaction F (6, 36) = 1.185 P=0.3361) EF1α liver: duration F (2, 34) = 2.751 P=0.0781, condition F (3, 34) = 1.322 P=0.2833 and their interaction F (6, 34) = 0.2528 P=0.9548.

In the binary mixture Cu and Cd, β-actin gills: interaction F (6, 36) = 1.089 P=0.3874, duration F (2, 36) = 1.450 P=0.2479; condition F (3, 36) = 1.215 P=0.3182; EF1α gills: interaction F (6, 36) = 1.148 P=0.3554, duration F (2, 36) = 0.9715 P=0.3882, condition F (3, 36) = 0.9569 P=0.4236; β-actin liver: interaction F (6, 36) = 2.353 P=0.0509; duration F (2, 36) = 1.569 P=0.2222, condition F (3, 36) = 2.077 P=0.1204; EF1α liver: interaction F (6, 33) = 0.6829 P=0.6645, duration F (2, 33) = 2.819 P=0.0741, condition F (3, 33) = 0.6011 P=0.6189.

In the ternary mixture Cu, Zn and Cd, β-actin gills: duration F (4, 60) = 0.5358 P=0.7099; condition F (3, 60) = 0.3975 P=0.7553 and interaction F (12, 60) = 0.7665 P=0.6814; β-actin liver: duration F (4, 29) = 1.318 P=0.2867; condition F (1, 29) = 0.3148 P=0.5791 and interaction F (4, 29) = 0.6802 P=0.6113; F (4, 30) = 0.7038 P=0.5955; EF1α gills: duration F (4, 28) = 2.245 P=0.0896; condition F (1, 28) = 1.290 P=0.2656 and their interaction F (4, 28) = 1.048 P=0.4006; EF1α Liver: duration F (4, 28) = 2.659 P=0.0535; condition F (4, 28) = 2.245 P=0.0896 and their interaction F (1, 28) = 0.6636 P=0.4222.

II. Relationship between metal content and MT expression

The relationship between metal content and MT expression in the gills and in the liver was examined by means of Pearson correlation. The correlation coefficients were calculated separately for each fish group (Kovarova et al. 2009). The tests were considered significant when P <0.05. Data analyses were performed using GraphPad Prism version 9.1 for Windows (GraphPad Software, La Jolla California USA).

Appendix table I: relationship between metal levels and expression of the gene coding for MT in the gill tissue.

Gills	Day 1		Day 3		Day 7	
	MT		MT		MT	
	Pearson r	P value	Pearson r	P value	Pearson r	P value
$Cu_{25\%}$	-0.62	0.38	-0.11	0.89	0.83	0.17
$Cu_{50\%}$	0.18	0.82	0.14	0.86	0.04	0.96
$Cu_{100\%}$	0.47	0.53	0.69	0.31	0.51	0.49
$Zn_{25\%}$	0.19	0.81	0.65	0.35	0.64	0.36
$Zn_{50\%}$	-0.69	0.31	-0.42	0.58	-0.72	0.28
$Zn_{100\%}$	-0.51	0.49	-0.67	0.33	0.66	0.54
$Cd_{25\%}$	0.95	0.05	0.91	0.09	0.65	0.35
$Cd_{50\%}$	0.35	0.65	-0.1	0.9	0.74	0.26
$Cd_{100\%}$	0.19	0.81	-0.74	0.26	0.95	0.05
Cu levels - Cu_{fix}/Cd_{10}	0.73	0.27	-0.27	0.73	-0.62	0.38
Cu levels - Cu_{fix}/Cd_{25}	0.51	0.49	-0.66	0.34	0.98*	0.02
Cu levels - Cu_{fix}/Cd_{50}	0.21	0.79	0.06	0.94	0.83	0.17
Cu levels - Cd_{fix}/Cu_{10}	0.69	0.31	0.76	0.24	0.98*	0.02
Cu levels - Cd_{fix}/Cu_{25}	-0.25	0.75	-0.32	0.68	0.96*	0.04
Cu levels - Cd_{fix}/Cu_{50}	0.6	0.4	0.55	0.45	0.36	0.64
Cd levels - Cu_{fix}/Cd_{10}	0.07	0.93	0.02	0.98	-0.58	0.42
Cd levels - Cu_{fix}/Cd_{25}	-0.27	0.73	-0.46	0.54	0.98*	0.02
Cd levels - Cu_{fix}/Cd_{50}	0.37	0.63	0.04	0.96	-0.85	0.35
Cd levels - Cd_{fix}/Cu_{10}	0.14	0.86	-0.22	0.78	0.44	0.56
Cd levels - Cd_{fix}/Cu_{25}	-0.07	0.93	0.04	0.96	0.97*	0.03
Cd levels - Cd_{fix}/Cu_{50}	0.32	0.68	0.69	0.31	0.35	0.65
Zn levels - Zn_{fix}/Cd_{10}	0.18	0.89	0.11	0.89	-0.19	0.81
Zn levels - Zn_{fix}/Cd_{25}	0.88	0.31	0.75	0.25	-0.48	0.52
Zn levels - Zn_{fix}/Cd_{50}	0.94	0.22	0.74	0.26	0.67	0.33
Zn levels - Cd_{fix}/Zn_{10}	-0.97*	0.03	0.76	0.24	0.78	0.22
Zn levels - Cd_{fix}/Zn_{25}	0.99	0.11	0.07	0.93	-0.5	0.5
Zn levels - Cd_{fix}/Zn_{50}	0.99	0.09	0.92	0.08	-0.26	0.74
Cd levels - Zn_{fix}/Cd_{10}	0.22	0.86	0.94	0.06	0.87	0.13
Cd levels - Zn_{fix}/Cd_{25}	-0.78	0.43	0.87	0.13	0.25	0.75
Cd levels - Zn_{fix}/Cd_{50}	0.52	0.65	-0.53	0.47	0.71	0.29
Cd levels - Cd_{fix}/Zn_{10}	-0.38	0.62	0.22	0.78	0.78	0.22
Cd levels - Cd_{fix}/Zn_{25}	-0.54	0.63	0.82	0.18	0.26	0.74
Cd levels - Cd_{fix}/Zn_{50}	-1*	0.02	0.92	0.08	0.77	0.23
Cu levels - $Cu_{10}/Zn_{10}/Cd_{10}$ (short term)	0.25	0.75	-0.47	0.53	-0.01	0.99
Zn levels - $Cu_{10}/Zn_{10}/Cd_{10}$ (short term)	0.61	0.39	0.36	0.64	-0.49	0.4
Cd levels - $Cu_{10}/Zn_{10}/Cd_{10}$ (short term)	0.02	0.98	-0.36	0.64	0.83	0.08

Appendix table II: relationship between metal levels and expression of the gene coding for MT in the liver tissue.

Liver	Day 1		Day 3		Day 7	
	MT		MT		MT	
	Pearson r	P value	Pearson r	P value	Pearson r	P value
Cu levels - Cu_{fix}/Cd_{10}	0.93	0.07	0.52	0.48	0.99*	0.01
Cu levels - Cu_{fix}/Cd_{25}	-0.25	0.75	-0.67	0.33	0.79	0.21
Cu levels - Cu_{fix}/Cd_{50}	-0.42	0.58	0.06	0.94	-0.19	0.81
Cu levels - Cd_{fix}/Cu_{10}	-0.34	0.66	0.29	0.71	-0.74	0.26
Cu levels - Cd_{fix}/Cu_{25}	-0.09	0.91	-0.57	0.43	0.74	0.26
Cu levels - Cd_{fix}/Cu_{50}	0.27	0.73	0.33	0.67	0.61	0.39
Cd levels - Cu_{fix}/Cd_{10}	0.3	0.7	0.24	0.76	0.16	0.84
Cd levels - Cu_{fix}/Cd_{25}	-0.42	0.58	-0.59	0.41	-0.97*	0.03
Cd levels - Cu_{fix}/Cd_{50}	-0.88	0.12	-0.95	0.05	0.76	0.24
Cd levels - Cd_{fix}/Cu_{10}	-0.97*	0.03	0.07	0.93	-0.76	0.24
Cd levels - Cd_{fix}/Cu_{25}	-0.53	0.47	0.19	0.81	-0.98	0.02
Cd levels - Cd_{fix}/Cu_{50}	0.36	0.64	-0.08	0.92	0.32	0.68
Zn levels - Zn_{fix}/Cd_{10}	-0.58	0.42	-0.22	0.78	0.89	0.11
Zn levels - Zn_{fix}/Cd_{25}	-0.24	0.76	0.39	0.61	0.24	0.76
Zn levels - Zn_{fix}/Cd_{50}	-0.9	0.1	1*	0.00	0.72	0.28
Zn levels - Cd_{fix}/Zn_{10}	-0.13	0.87	-0.52	0.48	-0.06	0.94
Zn levels - Cd_{fix}/Zn_{25}	-0.04	0.96	0.89	0.11	0.21	0.79
Zn levels - Cd_{fix}/Zn_{50}	0.93	0.07	0.92	0.08	-0.62	0.57
Cd levels - Zn_{fix}/Cd_{10}	0.21	0.79	0.51	0.49	0.43	0.57
Cd levels - Zn_{fix}/Cd_{25}	0.06	0.94	-0.58	0.42	-0.59	0.41
Cd levels - Zn_{fix}/Cd_{50}	0.47	0.53	0.07	0.93	-0.7	0.3
Cd levels - Cd_{fix}/Zn_{10}	-0.93	0.07	-0.33	0.67	-0.86	0.14
Cd levels - Cd_{fix}/Zn_{25}	-0.3	0.7	0.85	0.15	-0.58	0.42
Cd levels - Cd_{fix}/Zn_{50}	1*	0.0	0.88	0.12	0.58	0.61

Appendix table III: relationship between metal levels and the expression of the gene coding for MT in both the gill and the liver of common carp exposed to a ternary metal mixture and at two different temperatures.

Gills	10 ºC		20 ºC	
	MT		MT	
Day 1	Pearson r	P value	Pearson r	P value
$Cu_{10\%}$	0.31	0.69	0.75	0.25
$Zn_{10\%}$	0.35	0.65	-0.5	0.5
$Cd_{10\%}$	0.05	0.95	0.84	0.16
Day 7				
$Cu_{10\%}$	0.91	0.09	-0.43	0.57
$Zn_{10\%}$	0.74	0.26	0.47	0.53
$Cd_{10\%}$	0.32	0.68	0.31	0.69
Day 14				
$Cu_{10\%}$	0.8	0.2	0.6	0.4
$Zn_{10\%}$	-0.77	0.23	0.83	0.17
$Cd_{10\%}$	-0.33	0.67	0.5	0.5
Day 21				
$Cu_{10\%}$	-0.12	0.88	-0.35	0.65
$Zn_{10\%}$	0.48	0.48	-0.2	0.8
$Cd_{10\%}$	0.95	0.05	-0.8 *	0.2
Day 27				
$Cu_{10\%}$	-0.4	0.6	0.56	0.44
$Zn_{10\%}$	0.17	0.83	-0.82	0.18
$Cd_{10\%}$	0.66	0.34	0.83	0.17

Liver	10 ºC		20 ºC	
	MT		MT	
Day 1	Pearson r	P value	Pearson r	P value
$Cu_{10\%}$	-0.49	0.51	-0.86	0.14
$Zn_{10\%}$	0.01	0.99	0.1	0.9
$Cd_{10\%}$	-0.66	0.34	-0.36	0.64
Day 7				
$Cu_{10\%}$	0.26	0.74	-0.94	0.06
$Zn_{10\%}$	0.64	0.36	-0.92	0.08
$Cd_{10\%}$	-0.05	0.95	-0.56	0.44
Day 14				
$Cu_{10\%}$	0.94	0.06	-0.19	0.81
$Zn_{10\%}$	0.3	0.7	0.48	0.52
$Cd_{10\%}$	0.31	0.69	-0.25	0.75
Day 21				
$Cu_{10\%}$	-0.69	0.31	-0.4	0.6
$Zn_{10\%}$	-0.37	0.63	0.61	0.39
$Cd_{10\%}$	-0.47	0.53	-0.53	0.47
Day 27				
$Cu_{10\%}$	0.15	0.85	0.79	0.21
$Zn_{10\%}$	0.44	0.56	0.92	0.92
$Cd_{10\%}$	0.72	0.28	0.15	0.85

Appendix table IV: relationship between net metal levels and the expression of the gene coding for MT in the gill tissue

Net accumulated metals in the gills	Day 1		Day 3		Day 7	
	MT		MT		MT	
	Pearson r	P value	Pearson r	P value	Pearson r	P value
$Cu_{25\%}$	-0.62	0.38	-0.11	0.89	0.83	0.17
$Cu_{50\%}$	0.18	0.82	0.14	0.86	0.04	0.96
$Cu_{100\%}$	-0.76	0.53	0.69	0.31	0.51	0.49
$Zn_{25\%}$	0.19	0.81	0.65	0.35	0.64	0.36
$Zn_{50\%}$	-0.69	0.31	-0.42	0.58	-0.72	0.28
$Zn_{100\%}$	-0.51	0.49	-0.67	0.33	0.97*	0.03
$Cd_{25\%}$	0.95	0.05	0.91	0.09	0.65	0.35
$Cd_{50\%}$	0.35	0.65	-0.1	0.9	0.74	0.26
$Cd_{100\%}$	0.19	0.81	-0.74	0.26	0.95	0.05
Cu levels - Cu_{fix}/Cd_{10}	0.73	0.27	-0.27	0.73	-0.62	0.38
Cu levels - Cu_{fix}/Cd_{25}	0.51	0.49	-0.66	0.34	0.98*	0.02
Cu levels - Cu_{fix}/Cd_{50}	0.21	0.79	0.06	0.94	0.83	0.17
Cu levels - Cd_{fix}/Cu_{10}	0.69	0.31	0.76	0.24	0.98*	0.02
Cu levels - Cd_{fix}/Cu_{25}	-0.25	0.75	-0.32	0.68	0.96*	0.04
Cu levels - Cd_{fix}/Cu_{50}	0.6	0.4	0.55	0.45	0.36	0.64
Cd levels - Cu_{fix}/Cd_{10}	0.07	0.93	0.02	0.98	-0.58	0.42
Cd levels - Cu_{fix}/Cd_{25}	-0.27	0.73	-0.46	0.54	0.98*	0.02
Cd levels - Cu_{fix}/Cd_{50}	0.37	0.63	0.04	0.96	-0.85	0.35
Cd levels - Cd_{fix}/Cu_{10}	0.14	0.86	-0.22	0.78	0.44	0.56
Cd levels - Cd_{fix}/Cu_{25}	-0.07	0.93	0.04	0.96	0.97*	0.03
Cd levels - Cd_{fix}/Cu_{50}	0.32	0.68	0.69	0.31	0.35	0.65
Zn levels - Zn_{fix}/Cd_{10}	0.18	0.89	0.11	0.89	-0.19	0.81
Zn levels - Zn_{fix}/Cd_{25}	0.88	0.31	0.75	0.25	-0.48	0.52
Zn levels - Zn_{fix}/Cd_{50}	0.94	0.22	0.74	0.26	0.67	0.33
Zn levels - Cd_{fix}/Zn_{10}	-0.97	0.03	-0.11	0.89	0.78	0.22
Zn levels - Cd_{fix}/Zn_{25}	0.99	0.11	0.67	0.33	-0.5	0.5
Zn levels - Cd_{fix}/Zn_{50}	0.99	0.09	0.84	0.16	-0.26	0.74
Cd levels - Zn_{fix}/Cd_{10}	0.22	0.86	0.94	0.06	0.87	0.13
Cd levels - Zn_{fix}/Cd_{25}	-0.78	0.43	0.87	0.13	0.25	0.75
Cd levels - Zn_{fix}/Cd_{50}	0.52	0.65	-0.53	0.47	0.71	0.29
Cd levels - Cd_{fix}/Zn_{10}	-0.38	0.62	0.22	0.78	0.78	0.22
Cd levels - Cd_{fix}/Zn_{25}	-0.54	0.63	0.82	0.18	0.26	0.74
Cd levels - Cd_{fix}/Zn_{50}	-1*	0.02	0.92	0.08	0.77	0.23
Cu levels - $Cu_{10}/Zn_{10}/Cd_{10}$ (short term)	0.25	0.75	-0.47	0.53	-0.01	0.99
Zn levels - $Cu_{10}/Zn_{10}/Cd_{10}$ (short term)	0.61	0.39	0.36	0.64	-0.49	0.4
Cd levels - $Cu_{10}/Zn_{10}/Cd_{10}$ (short term)	0.02	0.98	-0.36	0.64	0.83	0.08

Appendix table V: relationship between net metal levels and the expression of the gene coding for MT in the liver tissue

Net accumulated metals in the liver	Day 1		Day 3		Day 7	
	MT		MT		MT	
	Pearson r	P value	Pearson r	P value	Pearson r	P value
Cu levels - Cu_{fix}/Cd_{10}	0.93	0.07	0.52	0.48	0.99*	0.01
Cu levels - Cu_{fix}/Cd_{25}	-0.25	0.75	-0.67	0.33	0.79	0.21
Cu levels - Cu_{fix}/Cd_{50}	-0.42	0.58	0.06	0.94	-0.19	0.81
Cu levels - Cd_{fix}/Cu_{10}	-0.34	0.66	0.29	0.71	-0.74	0.26
Cu levels - Cd_{fix}/Cu_{25}	-0.09	0.91	-0.57	0.43	0.74	0.26
Cu levels - Cd_{fix}/Cu_{50}	0.27	0.73	0.33	0.67	0.61	0.39
Cd levels - Cu_{fix}/Cd_{10}	0.3	0.7	0.24	0.76	0.16	0.84
Cd levels - Cu_{fix}/Cd_{25}	-0.42	0.58	-0.59	0.41	-0.97*	0.03
Cd levels - Cu_{fix}/Cd_{50}	-0.88	0.12	-0.95*	0.048	0.76	0.24
Cd levels - Cd_{fix}/Cu_{10}	-0.97*	0.03	0.07	0.93	-0.76	0.24
Cd levels - Cd_{fix}/Cu_{25}	-0.53	0.47	0.19	0.81	-0.98*	0.02
Cd levels - Cd_{fix}/Cu_{50}	0.36	0.64	-0.08	0.92	0.32	0.68
Zn levels - Zn_{fix}/Cd_{10}	-0.58	0.42	-0.22	0.78	0.89	0.11
Zn levels - Zn_{fix}/Cd_{25}	-0.24	0.76	0.39	0.61	0.24	0.76
Zn levels - Zn_{fix}/Cd_{50}	-0.9	0.1	1*	0.00	0.72	0.28
Zn levels - Cd_{fix}/Zn_{10}	-0.13	0.87	-0.52	0.48	-0.06	0.94
Zn levels - Cd_{fix}/Zn_{25}	-0.04	0.96	0.89	0.11	0.21	0.79
Zn levels - Cd_{fix}/Zn_{50}	0.93	0.07	0.92	0.08	-0.62	0.57
Cd levels - Zn_{fix}/Cd_{10}	0.21	0.79	0.51	0.49	0.43	0.57
Cd levels - Zn_{fix}/Cd_{25}	0.06	0.94	-0.58	0.42	-0.59	0.41
Cd levels - Zn_{fix}/Cd_{50}	0.47	0.53	0.07	0.93	-0.7	0.3
Cd levels - Cd_{fix}/Zn_{10}	-0.93	0.07	-0.33	0.67	-0.86	0.14
Cd levels - Cd_{fix}/Zn_{25}	-0.3	0.7	0.85	0.15	-0.58	0.42
Cd levels - Cd_{fix}/Zn_{50}	1*	0.00	0.88	0.12	0.58	0.61

Appendix table VI: relationship between metal levels (nmol/g or µmol/g) and the expression of the gene coding for MT in both the gill and the liver of common carp exposed to a ternary metal mixture and at two different temperatures

Net accumulated metals in the gills	10 ºC		20 ºC	
	MT		MT	
Day 1	Pearson r	P value	Pearson r	P value
$Cu_{10\%}$	0.31	0.69	0.75	0.25
$Zn_{10\%}$	0.35	0.65	-0.5	0.5
$Cd_{10\%}$	0.05	0.95	0.84	0.16
Day 7				
$Cu_{10\%}$	0.91	0.09	-0.43	0.57
$Zn_{10\%}$	0.74	0.26	0.47	0.53
$Cd_{10\%}$	0.32	0.68	0.31	0.69
Day 14				
$Cu_{10\%}$	0.8	0.2	0.6	0.4
$Zn_{10\%}$	-0.77	0.23	0.83	0.17
$Cd_{10\%}$	-0.33	0.67	0.5	0.5
Day 21				
$Cu_{10\%}$	-0.12	0.88	-0.35	0.65
$Zn_{10\%}$	0.48	0.52	-0.2	0.8
$Cd_{10\%}$	0.95	0.05	-0.8	0.2
Day 27				
$Cu_{10\%}$	-0.4	0.6	0.56	0.44
$Zn_{10\%}$	0.17	0.83	-0.82	0.18
$Cd_{10\%}$	0.66	0.34	0.83	0.17

Net accumulated metals in the liver	10 ºC		20 ºC	
	MT		MT	
Day 1	Pearson r	P value	Pearson r	P value
$Cu_{10\%}$	-0.49	0.51	-0.86	0.14
$Zn_{10\%}$	0.01	0.99	0.1	0.9
$Cd_{10\%}$	-0.66	0.34	-0.36	0.64
Day 7				
$Cu_{10\%}$	0.26	0.74	-0.94	0.06
$Zn_{10\%}$	0.64	0.36	-0.92	0.08
$Cd_{10\%}$	-0.05	0.95	-0.56	0.44
Day 14				
$Cu_{10\%}$	0.94	0.06	-0.19	0.81
$Zn_{10\%}$	0.3	0.7	0.48	0.52
$Cd_{10\%}$	0.31	0.69	-0.25	0.75
Day 21				
$Cu_{10\%}$	-0.69	0.31	-0.4	0.6
$Zn_{10\%}$	-0.37	0.63	0.61	0.39
$Cd_{10\%}$	-0.47	0.53	-0.53	0.47
Day 27				
$Cu_{10\%}$	0.15	0.85	0.79	0.21
$Zn_{10\%}$	0.44	0.56	-0.08	0.92
$Cd_{10\%}$	0.72	0.28	0.15	0.85

Bibliography

Abdel-Tawwab, M., and M. Wafeek. 2014. Influence of water temperature and waterborne cadmium toxicity on growth performance and metallothionein–cadmium distribution in different organs of Nile tilapia, *Oreochromis niloticus* (L.). Journal of thermal biology **45**:157-162.

Abdel-Tawwab, M., and M. Wafeek. 2017. Fluctuations in water temperature affected waterborne cadmium toxicity: Hematology, anaerobic glucose pathway, and oxidative stress status of Nile tilapia, *Oreochromis niloticus* (L.). Aquaculture **477**:106-111.

Al-Attar, A. M. 2005. Biochemical effects of short-term cadmium exposure on the freshwater fish, *Oreochromis niloticus*. Journal of Biological Sciences **5**:260-265.

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

Alam, M., and O. Maughan. 1992. The effect of malathion, diazinon, and various concentrations of zinc, copper, nickel, lead, iron, and mercury on fish. Biological trace element research **34**:225-236.

Ali, D., and H. Ali. 2015. Susceptibility of the freshwater pulmonate snail *Lymnea luteola* L. to copper oxide nanoparticle. Toxicological & Environmental Chemistry **97**:576-587.

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

Alsop, D. H., and C. M. Wood. 1999. Influence of waterborne cations on zinc uptake and toxicity in rainbow trout, *Oncorhynchus mykiss*. Canadian Journal of Fisheries and Aquatic Sciences **56**:2112-2119.

Altenburger, R., H. Walter, and M. Grote. 2004. What contributes to the combined effect of a complex mixture? Environmental science & technology **38**:6353-6362.

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

Amiard-Triquet, C., and J.-C. Amiard. 1998. Influence of ecological factors on accumulation of metal mixtures. Pages 351-386 Metal metabolism in aquatic environments. Springer.

Amiard, J.-C., C. Amiard-Triquet, S. Barka, J. Pellerin, and P. Rainbow. 2006. Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use as biomarkers. Aquatic Toxicology **76**:160-202.

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

Arini, A., P. Gourves, P. Gonzalez, and M. Baudrimont. 2015. Metal detoxification and gene expression regulation after a Cd and Zn contamination: an experimental study on *Danio rerio*. Chemosphere **128**:125-133.

Atli, G., and M. Canli. 2007. Enzymatic responses to metal exposures in a freshwater fish *Oreochromis niloticus*. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **145**:282-287.

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

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

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

Bagenal, T. 1978. Methods for assessment of fish production in fresh waters.

Baker, R., M. Knittel, and J. Fryer. 1983. Susceptibility of chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), and rainbow trout, *Salmo gairdneri* Richardson, to infection with *Vibrio anguillarum* following sublethal copper exposure. Journal of Fish Diseases **6**:267-275.

Begum, A., M. N. Amin, S. Kaneco, and K. Ohta. 2005. Selected elemental composition of the muscle tissue of three species of fish, *Tilapia nilotica*, *Cirrhina mrigala* and *Clarius batrachus*, from the fresh water Dhanmondi Lake in Bangladesh. Food chemistry **93**:439-443.

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

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

Bervoets, L., and R. Blust. 1999. Bioavailability of cadmium and zinc to midge larvae under natural and experimental conditions: effects of some environmental factors. Belgian Journal of Zoology **129**:269-284.

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

Bervoets, L., G. Knaepkens, M. Eens, and R. Blust. 2005. Fish community responses to metal pollution. Environmental Pollution **138**:338-349.

Bervoets, L., G. Meregalli, W. De Cooman, B. Goddeeris, and R. Blust. 2004. Caged midge larvae (*Chironomus riparius*) for the assessment of metal bioaccumulation from sediments in situ. Environmental Toxicology and Chemistry: An International Journal **23**:443-454.

Bervoets, L., K. Van Campenhout, H. Reynders, D. Knapen, A. Covaci, and R. Blust. 2009. Bioaccumulation of micropollutants and biomarker responses in caged carp (*Cyprinus carpio*). Ecotoxicology and Environmental Safety **72**:720-728.

Beyers, D. W., and M. S. Farmer. 2001. Effects of copper on olfaction of Colorado pikeminnow. Environmental Toxicology and Chemistry: An International Journal **20**:907-912.

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

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

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

Bolger, T., and P. Connolly. 1989. The selection of suitable indices for the measurement and analysis of fish condition. Journal of fish Biology **34**:171-182.

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

Bouchard, P., and H. Guderley. 2003. Time course of the response of mitochondria from oxidative muscle during thermal acclimation of rainbow trout, *Oncorhynchus mykiss*. Journal of Experimental Biology **206**:3455-3465.

Bourdineaud, J.-P., M. Baudrimont, P. Gonzalez, and J.-L. Moreau. 2006. Challenging the model for induction of metallothionein gene expression. Biochimie **88**:1787-1792.

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

Braz-Mota, S., L. M. L. Fé, F. A. C. Delunardo, H. Sadauskas-Henrique, V. M. F. de Almeida-Val, and A. L. Val. 2017. Exposure to waterborne copper and high temperature induces the formation of reactive oxygen species and causes

mortality in the Amazonian fish *Hoplosternum littorale*. Hydrobiologia **789**:157-166.

Brett, J. R. 1971. Energetic responses of salmon to temperature. A study of some thermal relations in the physiology and freshwater ecology of sockeye salmon (*Oncorhynchus nerkd*). American Zoologist **11**:99-113.

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

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

Brown, M. W., D. G. Thomas, D. Shurben, J. Solbe, J. Kay, and A. Cryer. 1986. A comparison of the differential accumulation of cadmium in the tissues of three species of freshwater fish, *Salmo gairdneri*, *Rutilus rutilus* and *Noemacheilus barbatulus*. Comparative biochemistry and physiology. C, Comparative pharmacology and toxicology **84**:213-217.

Brungs, W. A., E. N. Leonard, and J. M. McKim. 1973. Acute and long-term accumulation of copper by the brown bullhead, *Ictalurus nebulosus*. Journal of the Fisheries Board of Canada **30**:583-586.

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

Buckley, J., M. Roch, J. McCarter, C. Rendell, and A. Matheson. 1982. Chronic exposure of coho salmon to sublethal concentrations of copper.--I. Effect on growth, on accumulation and distribution of copper, and on copper tolerance. Comparative biochemistry and physiology. C: Comparative pharmacology **72**:15-19.

Bury, N. R., and M. Grosell. 2003. Waterborne iron acquisition by a freshwater teleost fish, zebrafish *Danio rerio*. Journal of Experimental Biology **206**:3529-3535.

Bury, N. R., P. A. Walker, and C. N. Glover. 2003. Nutritive metal uptake in teleost fish. Journal of Experimental Biology **206**:11-23.

Bustin, S. A., V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, and G. L. Shipley. 2009. The MIQE Guidelines: M inimum I nformation for Publication of Q uantitative Real-Time PCR E xperiments. Oxford University Press.

Cairns, J., A. G. Heath, and B. C. Parker. 1975. The effects of temperature upon the toxicity of chemicals to aquatic organisms. Hydrobiologia **47**:135-171.

Carvalho, C., H. S. S. de Araujo, and M. N. Fernandes. 2004. Hepatic metallothionein in a teleost (*Prochilodus scrofa*) exposed to copper at pH 4.5 and pH 8.0. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology **137**:225-234.

Carvalho, C., and M. Fernandes. 2006. Effect of temperature on copper toxicity and hematological responses in the neotropical fish *Prochilodus scrofa* at low and high pH. Aquaculture **251**:109-117.

Casalino, E., G. Calzaretti, C. Sblano, and C. Landriscina. 2002. Molecular inhibitory mechanisms of antioxidant enzymes in rat liver and kidney by cadmium. Toxicology **179**:37-50.

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

Castaldo, G., V. Delahaut, B. Slootmaekers, L. Bervoets, R. M. Town, R. Blust, and G. De Boeck. 2020a. 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:In press.

Castaldo, G., G. Flipkens, M. Pillet, R. Town, L. Bervoets, R. Blust, and G. De Boeck. 2020b. Antagonistic bioaccumulation of waterborne Cu (II) and Cd (II) in common carp (*Cyprinus carpio*) and effects on ion-homeostasis and defensive mechanisms. Aquatic Toxicology **226**:105561.

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

Caulton, M. 1978. The effect of temperature and mass on routine metabolism in *Sarotherodon* (Tilapia) *mossambicus* (Peters). Journal of fish Biology **13**:195-201.

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

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

Chan, P. C., C. K. Shiu, F. W.-Y. Wong, J. K.-Y. Wong, K. L. Lam, and K. M. Chan. 2004. Common carp metallothionein-1 gene: cDNA cloning, gene structure and expression studies. Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression **1676**:162-171.

Chatzidimitriou, E., P. Bisaccia, F. Corrà, M. Bonato, P. Irato, L. Manuto, S. Toppo, R. Bakiu, and G. Santovito. 2020. Copper/zinc superoxide dismutase from the crocodile icefish *Chionodraco hamatus*: Antioxidant defense at constant sub-zero temperature. Antioxidants **9**:325.

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

Choi, C. Y., K. W. An, E. R. Nelson, and H. R. Habibi. 2007. Cadmium affects the expression of metallothionein (MT) and glutathione peroxidase (GPX) mRNA in goldfish, *Carassius auratus*. Comparative Biochemistry and Physiology **145**:595-600.

Chowdhury, M., B. Baldisserotto, and C. Wood. 2005. Tissue-specific cadmium and metallothionein levels in rainbow trout chronically acclimated to waterborne or dietary cadmium. Archives of Environmental Contamination and Toxicology **48**:381-390.

Chowdhury, M., D. McDonald, and C. Wood. 2004. Gastrointestinal uptake and fate of cadmium in rainbow trout acclimated to sublethal dietary cadmium. Aquatic Toxicology **69**:149-163.

Chowdhury, M. J., M. Grosell, D. McDonald, and C. Wood. 2003. Plasma clearance of cadmium and zinc in non-acclimated and metal-acclimated trout. Aquatic Toxicology **64**:259-275.

Chowdhury, M. J., and C. M. Wood. 2007. Renal function in the freshwater rainbow trout after dietary cadmium acclimation and waterborne cadmium challenge. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **145**:321-332.

Chung, M. J., P. A. Walker, R. W. Brown, and C. Hogstrand. 2005. ZINC-mediated gene expression offers protection against H2O2-induced cytotoxicity. Toxicology and applied pharmacology **205**:225-236.

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

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

Cocchio, L., F. Beamish, and D. Rodgers. 1995. Effects of water chemistry and temperature on radiocesium dynamics in rainbow trout, *Oncorhynchus mykiss*. Canadian Journal of Fisheries and Aquatic Sciences **52**:607-613.

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

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

Cowey, C., and J. Sargent. 1972. Fish nutrition. Advances in marine biology **10**:383-494.

Coyle, P., J. Philcox, L. Carey, and A. Rofe. 2002. Metallothionein: the multipurpose protein. Cellular and Molecular Life Sciences CMLS **59**:627-647.

Craig, P. M., M. Galus, C. M. Wood, and G. B. McClelland. 2009. Dietary iron alters waterborne copper-induced gene expression in soft water acclimated zebrafish (*Danio rerio*). American Journal of Physiology-Regulatory, Integrative and Comparative Physiology **296**:R362-R373.

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

Crain, C. M., K. Kroeker, and B. S. Halpern. 2008. Interactive and cumulative effects of multiple human stressors in marine systems. Ecology letters **11**:1304-1315.

Cretì, P., F. Trinchella, and R. Scudiero. 2010. Heavy metal bioaccumulation and metallothionein content in tissues of the sea bream *Sparus aurata* from three different fish farming systems. Environmental Monitoring and Assessment **165**:321-329.

Crossin, G. T., S. Hinch, S. Cooke, D. Welch, D. Patterson, S. Jones, A. Lotto, R. Leggatt, M. Mathes, and J. Shrimpton. 2008. Exposure to high temperature influences the behaviour, physiology, and survival of sockeye salmon during spawning migration. Canadian Journal of Zoology **86**:127-140.

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

Dalton, T. P., L. He, B. Wang, M. L. Miller, L. Jin, K. F. Stringer, X. Chang, C. S. Baxter, and D. W. Nebert. 2005. Identification of mouse SLC39A8 as the transporter responsible for cadmium-induced toxicity in the testis. Proceedings of the National Academy of Sciences **102**:3401-3406.

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

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

Dautremepuits, C., S. Paris-Palacios, S. Betoulle, and G. Vernet. 2004. Modulation in hepatic and head kidney parameters of carp (*Cyprinus carpio* L.) induced by copper and chitosan. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **137**:325-333.

De Boeck, G., M. Eyckmans, I. Lardon, R. Bobbaers, A. Sinha, and R. Blust. 2010a. Metal accumulation and metallothionein induction in the spotted dogfish *Scyliorhinus canicula*. Comparative Biochemistry Physiology **155**:503-508.

De Boeck, G., W. Meeus, W. De Coen, and R. Blust. 2004. Tissue-specific Cu bioaccumulation patterns and differences in sensitivity to waterborne Cu in three freshwater fish: rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*), and gibel carp (*Carassius auratus gibelio*). Aquatic Toxicology **70**:179-188.

De Boeck, G., T. T. H. Ngo, K. Van Campenhout, and R. Blust. 2003. Differential metallothionein induction patterns in three freshwater fish during sublethal copper exposure. Aquatic Toxicology **65**:413-424.

De Boeck, G., G. Nilsson, A. Vlaeminck, and R. Blust. 1996. Central monoaminergic responses to salinity and temperature rises in common carp. Journal of Experimental Biology **199**:1605-1611.

De Boeck, G., R. Smolders, and R. Blust. 2010b. Copper toxicity in gibel carp *Carassius auratus gibelio*: importance of sodium and glycogen. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **152**:332-337.

De Boeck, G., K. Van der Ven, W. Meeus, and R. Blust. 2007. Sublethal copper exposure induces respiratory stress in common and gibel carp but not in rainbow trout. Comparative Biochemistry Physiology **144**:380-390.

De Boeck, G., A. Vlaeminck, P. H. M. Balm, R. A. C. Lock, B. De Wachter, and R. Blust. 2001. Morphological and metabolic changes in common carp, *Cyprinus carpio*, during short-term copper exposure: Interactions between Cu2+ and plasma cortisol elevation. Environmental Toxicology and Chemistry **20**:374-381.

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

De Jonge, M., S. Lofts, L. Bervoets, and R. Blust. 2014. Relating metal exposure and chemical speciation to trace metal accumulation in aquatic insects under natural field conditions. Science of The Total Environment **496**:11-21.

De Smet, H., and R. Blust. 2001. Stress responses and changes in protein metabolism in carp *Cyprinus carpio* during cadmium exposure. Ecotoxicology and Environmental Safety **48**:255-262.

De Smet, H., B. De Wachter, R. Lobinski, and R. Blust. 2001. Dynamics of (Cd,Zn)-metallothioneins in gills, liver and kidney of common carp *Cyprinus carpio* during cadmium exposure. Aquatic Toxicology **52**:269-281.

Defo, M. A., L. Bernatchez, P. G. Campbell, and P. Couture. 2015. Transcriptional and biochemical markers in transplanted *Perca flavescens* to characterize cadmium-and copper-induced oxidative stress in the field. Aquatic Toxicology **162**:39-53.

Delahaut, V., O. Daelemans, A. K. Sinha, G. De Boeck, and L. Bervoets. 2019. A multibiomarker approach for evaluating environmental contamination: Common carp (*Cyprinus carpio*) transplanted along a gradient of metal pollution. Science of The Total Environment **669**:481-492.

Delahaut, V., B. Rašković, M. S. Salvado, L. Bervoets, R. Blust, and G. De Boeck. 2020. Toxicity and bioaccumulation of Cadmium, Copper and Zinc in a direct comparison at equitoxic concentrations in common carp (*Cyprinus carpio*) juveniles. PloS one **15**:e0220485.

Deshmukh, S., and V. Marathe. 1980. Size related toxicity of copper & mercury to *Lebistes reticulatus* (Peter), *Labeo rohita* (Ham.) & *Cyprinus carpio* Linn. Indian journal of experimental biology **18**:421.

Dethloff, G., H. Bailey, and K. Maier. 2001. Effects of dissolved copper on select hematological, biochemical, and immunological parameters of wild rainbow trout (*Oncorhynchus mykiss*). Archives of Environmental Contamination and Toxicology **40**:371-380.

Díaz-de-Alba, M., A. C. Raya, M. Granado-Castro, M. O. Ramírez, B. El Mai, F. C. García, M. Troyano-Montoro, E. Espada-Bellido, R. T. Santiago, and M. Galindo-Riaño. 2017. Biomarker responses of Cu-induced toxicity in European seabass *Dicentrarchus labrax*: Assessing oxidative stress and histopathological alterations. Marine pollution bulletin **124**:336-348.

Dickinson, D. A., and H. J. Forman. 2002. Cellular glutathione and thiols metabolism. Biochemical pharmacology **64**:1019-1026.

Dixon, D. G., and J. B. Sprague. 1981. Copper bioaccumulation and hepatoprotein synthesis during acclimation to copper by juvenile rainbow trout. Aquatic Toxicology **1**:69-81.

Dolomatov, S., W. Zukow, and R. Brudnicki. 2013. Role of temperature in regulation of the life cycle of temperate fish. Russian Journal of Marine Biology **39**:81-91.

Domingos, R. F., A. Gélabert, S. Carreira, A. Cordeiro, Y. Sivry, and M. F. Benedetti. 2015. Metals in the aquatic environment—interactions and implications for the speciation and bioavailability: a critical overview. Aquatic geochemistry **21**:231-257.

Douben, P. E. 1989. Uptake and elimination of waterborne cadmium by the fish *Noemacheilus barbatulus* L.(stone loach). Archives of Environmental Contamination and Toxicology **18**:576-586.

Driessnack, M. K., A. Jamwal, and S. Niyogi. 2017. Effects of chronic waterborne cadmium and zinc interactions on tissue-specific metal accumulation and reproduction in fathead minnow (*Pimephales promelas*). Ecotoxicology and Environmental Safety **140**:65-75.

Dymowska, A. K., P.-P. Hwang, and G. G. Goss. 2012. Structure and function of ionocytes in the freshwater fish gill. Respiratory physiology & neurobiology **184**:282-292.

Dziegiel, P., B. Pula, C. Kobierzycki, M. Stasiolek, and M. Podhorska-Okolow. 2016. Metallothioneins: structure and functions. Metallothioneins in Normal and Cancer Cells:3-20.

Ebrahimpour, M., H. Alipour, and S. Rakhshah. 2010. Influence of water hardness on acute toxicity of copper and zinc on fish. Toxicology and Industrial Health **26**:361-365.

Eisler, R. 1993. Zinc hazards to fish, wildlife, and invertebrates: a synoptic review. US Department of the Interior, Fish and Wildlife Service.

Eissa, B., N. Ossana, L. Ferrari, and A. Salibián. 2010. Quantitative behavioral parameters as toxicity biomarkers: fish responses to waterborne cadmium. Archives of Environmental Contamination and Toxicology **58**:1032-1039.

Elliott, N., R. Swain, and D. Ritz. 1986. Metal interaction during accumulation by the mussel *Mytilus edulis planulatus*. Marine Biology **93**:395-399.

EPA. 2018. https://www.epa.gov/wqc/national-recommended-water-quality-criteria-aquatic-life-criteria-table

EPA, U. S. E. P. A., Office of Water, Office of Science, and Technology. 2004. National recommended water quality criteria.

Eroglu, A., Z. Dogan, E. Kanak, G. Atli, and M. Canli. 2015. Effects of heavy metals (Cd, Cu, Cr, Pb, Zn) on fish glutathione metabolism. Environmental Science and Pollution Research **22**:3229-3237.

Eroglu, K., G. Atli, and M. Canli. 2005. Effects of metal (Cd, Cu, Zn) interactions on the profiles of metallothionein-like proteins in the Nile Fish. Bull. Environ. Contam. Toxicol **75**:390-399.

Espinoza, H. M., C. R. Williams, and E. P. Gallagher. 2012. Effect of cadmium on glutathione S-transferase and metallothionein gene expression in coho salmon liver, gill and olfactory tissues. Aquatic Toxicology **110**:37-44.

Evans, D. H., P. M. Piermarini, and K. P. Choe. 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiological reviews **85**:97-177.

Evans, J., and L. Hastings. 1992. Accumulation of Cd (II) in the CNS depending on the route of administration: intraperitoneal, intratracheal, or intranasal. Toxicological Sciences **19**:275-278.

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

Eyckmans, M., C. Tudorache, V. M. Darras, R. Blust, and G. De Boeck. 2010. Hormonal and ion regulatory response in three freshwater fish species following waterborne copper exposure. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **152**:270-278.

Fazelan, Z., S. M. Hoseini, M. Yousefi, M. Khalili, S. H. Hoseinifar, and H. Van Doan. 2020. Effects of dietary eucalyptol administration on antioxidant and inflammatory genes in common carp (*Cyprinus carpio*) exposed to ambient copper. Aquaculture **520**:734988.

Feeney, G. P., D. Zheng, P. Kille, and C. Hogstrand. 2005. The phylogeny of teleost ZIP and ZnT zinc transporters and their tissue specific expression and response to zinc in zebrafish. Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression **1732**:88-95.

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

Fenwick, J., S. W. Bonga, and G. Flik. 1999. In vivo bafilomycin-sensitive Na (+) uptake in young freshwater fish. Journal of Experimental Biology **202**:3659-3666.

Ferain, A., C. Bonnineau, I. Neefs, N. De Saeyer, B. Lemaire, V. Cornet, Y. Larondelle, K. A. De Schamphelaere, C. Debier, and J.-F. Rees. 2018. Exploring the interactions between polyunsaturated fatty acids and cadmium in rainbow

trout liver cells: a genetic and proteomic study. Aquatic Toxicology **205**:100-113.

Ferro, D., R. Bakiu, C. De Pittà, F. Boldrin, F. Cattalini, S. Pucciarelli, C. Miceli, and G. Santovito. 2015. Cu, Zn superoxide dismutases from *Tetrahymena thermophila*: molecular evolution and gene expression of the first line of antioxidant defenses. Protist **166**:131-145.

Ferro, D., R. Bakiu, S. Pucciarelli, C. Miceli, A. Vallesi, P. Irato, and G. Santovito. 2020. Molecular Characterization, Protein–Protein Interaction Network, and Evolution of Four Glutathione Peroxidases from *Tetrahymena thermophila*. Antioxidants **9**:949.

Fiess, J. C., A. Kunkel-Patterson, L. Mathias, L. G. Riley, P. H. Yancey, T. Hirano, and E. G. Grau. 2007. Effects of environmental salinity and temperature on osmoregulatory ability, organic osmolytes, and plasma hormone profiles in the Mozambique tilapia (*Oreochromis mossambicus*). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology **146**:252-264.

Figueira, E., D. Branco, S. C. Antunes, F. Gonçalves, and R. Freitas. 2012. Are metallothioneins equally good biomarkers of metal and oxidative stress? Ecotoxicology and Environmental Safety **84**:185-190.

Fine, M., D. Zilberg, Z. Cohen, G. Degani, B. Moav, and A. Gertler. 1996. The effect of dietary protein level, water temperature and growth hormone administration on growth and metabolism in the common carp (*Cyprinus carpio*). Comparative Biochemistry and Physiology Part A: Physiology **114**:35-42.

Fırat, Ö., H. Y. Cogun, S. Aslanyavrusu, and F. Kargın. 2009. Antioxidant responses and metal accumulation in tissues of Nile tilapia *Oreochromis niloticus* under Zn, Cd and Zn+ Cd exposures. Journal of Applied Toxicology **29**:295-301.

Franklin, N. M., J. L. Stauber, R. P. Lim, and P. Petocz. 2002. Toxicity of metal mixtures to a tropical freshwater alga (*Chlorella* sp.): the effect of interactions between copper, cadmium, and zinc on metal cell binding and uptake. Environmental Toxicology and Chemistry: An International Journal **21**:2412-2422.

Freedman, J. H., M. R. Ciriolo, and J. Peisach. 1989. The role of glutathione in copper metabolism and toxicity. Journal of Biological Chemistry **264**:5598-5605.

Fry, F. 1971. The effect of environmental factors on the physiology of fish. Fish physiology:1-98.

Fu, H., R. Lock, and S. W. Bonga. 1989. Effect of cadmium on prolactin cell activity and plasma electrolytes in the freshwater teleost *Oreochromis mossambicus*. Aquatic Toxicology **14**:295-306.

Fu, H., O. Steinebach, C. Van den Hamer, P. Balm, and R. Lock. 1990. Involvement of cortisol and metallothionein-like proteins in the physiological responses of tilapia (*Oreochromis mossambicus*) to sublethal cadmium stress. Aquatic Toxicology **16**:257-269.

Galvez, F., N. M. Franklin, R. B. Tuttle, and C. M. Wood. 2007. Interactions of waterborne and dietary cadmium on the expression of calcium transporters in the gills of rainbow trout: influence of dietary calcium supplementation. Aquatic Toxicology **84**:208-214.

Ganesh, N., T. Gupta, R. Katti, and K. Udupa. 2000. Acute toxicity of copper on three life stages of common carp, *Cyprinus carpio* Var. communis. Pollution Research **19**:91-93.

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

Gilmour, K., and S. Perry. 2009. Carbonic anhydrase and acid–base regulation in fish. Journal of Experimental Biology **212**:1647-1661.

Glynn, A. W. 2001. The influence of zinc on apical uptake of cadmium in the gills and cadmium influx to the circulatory system in zebrafish (*Danio rerio*). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **128**:165-172.

Gonzalez, R., and D. McDonald. 2000. Ionoregulatory responses to temperature change in two species of freshwater fish. Fish physiology and biochemistry **22**:311-317.

Green, W. W., R. S. Mirza, C. M. Wood, and G. G. Pyle. 2010. Copper binding dynamics and olfactory impairment in fathead minnows (*Pimephales promelas*). Environmental science & technology **44**:1431-1437.

Grosell, M. 2011. Copper. Pages 53-133 *in* C. M. Wood, A. P. Farrell, and C. J. Brauner, editors. Fish physiology. Academic Press.

Grosell, M., K. M. Gilmour, and S. F. Perry. 2007. Intestinal carbonic anhydrase, bicarbonate, and proton carriers play a role in the acclimation of rainbow trout to seawater. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology.

Grosell, M., H. J. Hansen, and P. Rosenkilde. 1998. Cu uptake, metabolism and elimination in fed and starved European eels (*Anguilla anguilla*) during adaptation to water-borne Cu exposure. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology **120**:295-305.

Grosell, M., C. Hogstrand, and C. Wood. 1997. Cu uptake and turnover in both Cu-acclimated and non-acclimated rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology **38**:257-276.

Grosell, M., M. McDonald, C. Wood, and P. Walsh. 2004. Effects of prolonged copper exposure in the marine gulf toadfish (*Opsanus beta*): I. Hydromineral balance and plasma nitrogenous waste products. Aquatic Toxicology **68**:249-262.

Grosell, M., J. McGeer, and C. M. Wood. 2001. Plasma copper clearance and biliary copper excretion are stimulated in copper-acclimated trout. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology **280**:R796-R806.

Grosell, M., C. Nielsen, and A. Bianchini. 2002. Sodium turnover rate determines sensitivity to acute copper and silver exposure in freshwater animals. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **133**:287-303.

Grosell, M., and C. M. Wood. 2002. Copper uptake across rainbow trout gills: mechanisms of apical entry. Journal of Experimental Biology **205**:1179-1188.

Grosell, M., C. M. Wood, and P. J. Walsh. 2003. Copper homeostasis and toxicity in the elasmobranch Raja erinacea and the teleost *Myoxocephalus octodecemspinosus* during exposure to elevated water-borne copper. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **135**:179-190.

Guderley, H. 2004. Metabolic responses to low temperature in fish muscle. Biological reviews **79**:409-427.

Guinot, D., R. Ureña, A. Pastor, I. Varó, J. Del Ramo, and A. Torreblanca. 2012. Long-term effect of temperature on bioaccumulation of dietary metals and metallothionein induction in *Sparus aurata*. Chemosphere **87**:1215-1221.

Guo, S.-N., J.-L. Zheng, S.-S. Yuan, and Q.-L. Zhu. 2018. Effects of heat and cadmium exposure on stress-related responses in the liver of female zebrafish: Heat increases cadmium toxicity. Science of The Total Environment **618**:1363-1370.

Hamilton, S. J., and P. M. Mehrle. 1986. Metallothionein in Fish - Review of Its Importance in Assessing Stress from Metal Contaminants. Transactions of the American Fisheries Society **115**:596-609.

Handeland, S. O., A. K. Imsland, and S. O. Stefansson. 2008. The effect of temperature and fish size on growth, feed intake, food conversion efficiency and stomach evacuation rate of Atlantic salmon post-smolts. Aquaculture **283**:36-42.

Handy, R. 1996. Dietary exposure to toxic metals in fish. Pages 29-60 *in* Seminar Series-Society for Experimental Biology. Cambridge University Press.

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

Handy, R. D. 2003. Chronic effects of copper exposure versus endocrine toxicity: two sides of the same toxicological process? Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology **135**:25-38.

Hansen, B., S. Rømma, Ø. Garmo, P. Olsvik, and R. Andersen. 2006. Antioxidative stress proteins and their gene expression in brown trout (*Salmo trutta*) from three rivers with different heavy metal levels. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **143**:263-274.

Hansen, B. H., S. Rømma, Ø. A. Garmo, S. A. Pedersen, P. A. Olsvik, and R. A. Andersen. 2007. Induction and activity of oxidative stress-related proteins during waterborne Cd/Zn-exposure in brown trout (*Salmo trutta*). Chemosphere **67**:2241-2249.

Hardy, R. W., C. V. Sullivan, and A. M. Koziol. 1987. Absorption, body distribution, and excretion of dietary zinc by rainbow trout (*Salmo gairdneri*). Fish physiology biochemistry **3**:133-143.

Hasanuzzaman, M., K. Nahar, T. I. Anee, and M. Fujita. 2017. Glutathione in plants: biosynthesis and physiological role in environmental stress tolerance. Physiology and Molecular Biology of Plants **23**:249-268.

Hashemi, S., R. Blust, and G. De Boeck. 2008a. The effect of starving and feeding on copper toxicity and uptake in Cu acclimated and non-acclimated carp. Aquatic Toxicology **86**:142-147.

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

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

Hassler, C. S., V. I. Slaveykova, and K. J. Wilkinson. 2004. Some fundamental (and often overlooked) considerations underlying the free ion activity and biotic ligand models. Environmental Toxicology and Chemistry: An International Journal **23**:283-291.

Heath, A. G. 1995. Water pollution and fish physiology. CRC press. INC. Boca. Raton, Florida. 356.

Hering, D., A. Borja, J. Carstensen, L. Carvalho, M. Elliott, C. K. Feld, A.-S. Heiskanen, R. K. Johnson, J. Moe, and D. Pont. 2010. The European Water Framework Directive at the age of 10: a critical review of the achievements with recommendations for the future. Science of The Total Environment **408**:4007-4019.

Hermesz, E., M. Ábrahám, and J. Nemcsók. 2001. Tissue-specific expression of two metallothionein genes in common carp during cadmium exposure and temperature shock. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **128**:457-465.

Hermesz, E., A. P. Gazdag, A. K. Said, J. Nemcsok, and M. Ábrahám. 2002. Differential regulation of the two metallothionein genes in common carp. Acta biologica Hungarica **53**:343-350.

Hochachka, P. W., and G. N. Somero. 2002. Biochemical adaptation: mechanism and process in physiological evolution. Oxford University Press.

Hodson, P. V., and J. B. Sprague. 1975. Temperature-induced changes in acute toxicity of zinc to Atlantic salmon (*Salmo salar*). Journal of the Fisheries Board of Canada **32**:1-10.

Hogstrand, C. 2011. Zinc. Pages 135-200 *in* C. M. Wood, A. P. Farrell, and C. J. Brauner, editors. Fish physiology. Academic Press.

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

Hogstrand, C., S. Reid, and C. Wood. 1995. Ca2+ versus Zn2+ transport in the gills of freshwater rainbow trout and the cost of adaptation to waterborne Zn2+. Journal of Experimental Biology **198**:337-348.

Hogstrand, C., P. M. Verbost, S. Bonga, and C. M. Wood. 1996. Mechanisms of zinc uptake in gills of freshwater rainbow trout: interplay with calcium transport. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology **270**:R1141-R1147.

Hogstrand, C., R. W. Wilson, D. Polgar, and C. M. Wood. 1994. Effects of zinc on the kinetics of branchial calcium uptake in freshwater rainbow trout during adaptation to waterborne zinc. Journal of Experimental Biology **186**:55-73.

Hogstrand, C., and C. Wood. 1996. The physiology and toxicology of zinc in fish. Pages 61-84 *in* Seminar series-society for experimental biology. Cambridge University Press.

Hogstrand, C., and C. M. Wood. 1995. Mechanisms for zinc acclimation in freshwater rainbow trout. Marine Environmental Research **39**:131-135.

Hogstrand, C., D. Zheng, G. Feeney, P. Cunningham, and P. Kille. 2008. Zinc-controlled gene expression by metal-regulatory transcription factor 1 (MTF1) in a model vertebrate, the zebrafish. Portland Press Limited.

Hollis, L., C. Hogstrand, and C. Wood. 2001. Tissue-specific cadmium accumulation, metallothionein induction, and tissue zinc and copper levels during chronic sublethal cadmium exposure in juvenile rainbow trout. Archives of Environmental Contamination and Toxicology **41**:468-474.

Hollis, L., J. C. McGeer, D. G. McDonald, and C. M. Wood. 1999. Cadmium accumulation, gill Cd binding, acclimation, and physiological effects during long term sublethal Cd exposure in rainbow trout. Aquatic Toxicology **46**:101-119.

Hollis, L., J. C. McGeer, D. G. McDonald, and C. M. Wood. 2000a. Effects of long term sublethal Cd exposure in rainbow trout during soft water exposure: implications for biotic ligand modelling. Aquatic Toxicology **51**:93-105.

Hollis, L., J. C. McGeer, D. G. McDonald, and C. M. Wood. 2000b. Protective effects of calcium against chronic waterborne cadmium exposure to juvenile rainbow trout. Environmental Toxicology and Chemistry: An International Journal **19**:2725-2734.

Honda, R. T., M. Fernandes-de-Castilho, and A. L. Val. 2008. Cadmium-induced disruption of environmental exploration and chemical communication in matrinxã, *Brycon amazonicus*. Aquatic Toxicology **89**:204-206.

Hopkin, S. P. 1989. Ecophysiology of metals in terrestrial invertebrates. Elsevier Applied Science Publishers.

Hwang, P.-P. 2009. Ion uptake and acid secretion in zebrafish (*Danio rerio*). Journal of Experimental Biology **212**:1745-1752.

Hwang, P.-P., and T.-H. Lee. 2007. New insights into fish ion regulation and mitochondrion-rich cells. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology **148**:479-497.

Hwang, P.-P., T.-H. Lee, and L.-Y. Lin. 2011. Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology **301**:R28-R47.

Hyllner, S. J., T. Andersson, C. Haux, and P. E. Olsson. 1989. Cortisol induction of metallothionein in primary culture of rainbow trout hepatocytes. Journal of cellular physiology **139**:24-28.

Illuminati, S., C. Truzzi, A. Annibaldi, B. Migliarini, O. Carnevali, and G. Scarponi. 2010. Cadmium bioaccumulation and metallothionein induction in the liver of the Antarctic teleost *Trematomus bernacchii* during an on-site short-term exposure to the metal via seawater. Toxicological & Environmental Chemistry **92**:617-640.

Isani, G., G. Andreani, F. Cocchioni, D. Fedeli, E. Carpene, and G. Falcioni. 2009. Cadmium accumulation and biochemical responses in Sparus aurata following sub-lethal Cd exposure. Ecotoxicology and Environmental Safety **72**:224-230.

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

Javed, M., and N. Usmani. 2019. An overview of the adverse effects of heavy metal contamination on fish health. Proceedings of the National Academy of Sciences, India Section B: Biological Sciences **89**:389-403.

Javid, A., M. Javed, and S. Abdullah. 2007. Nickel bio-accumulation in the bodies of *Catla catla*, *Labeo rohita* and *Cirrhina mrigala* during 96-hr LC50 exposures. Int. J. Agric. Biol **9**:139-142.

Jezierska, B., and M. Witeska. 2006. The metal uptake and accumulation in fish living in polluted waters. Pages 107-114 Soil and water pollution monitoring, protection and remediation. Springer.

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

Jiang, X., X. Guan, L. Yao, H. Zhang, X. Jin, and Y. Han. 2016. Effects of single and joint subacute exposure of copper and cadmium on heat shock proteins in common carp (*Cyprinus carpio*). Biological trace element research **169**:374-381.

Kalay, M. 2006. The Effect of Cadmium on the Levels of Na+, K+, Ca++ and Mg++ in Serum of *Tilapia nilotica* (Linnaeus, 1758). Ekoloji **15**:1-7.

Kalay, M., and M. Canli. 2000. Elimination of essential (Cu, Zn) and non-essential (Cd, Pb) metals from tissues of a freshwater fish *Tilapia zilli*. Turkish Journal of Zoology **24**:429-436.

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

Kamunde, C., M. Grosell, D. Higgs, and C. M. Wood. 2002b. Copper metabolism in actively growing rainbow trout (*Oncorhynchus mykiss*): interactions between dietary and waterborne copper uptake. Journal of Experimental Biology **205**:279-290.

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

Kamunde, C., and C. M. Wood. 2003. The influence of ration size on copper homeostasis during sublethal dietary copper exposure in juvenile rainbow trout, *Oncorhynchus mykiss*. Aquatic Toxicology **62**:235-254.

Kamunde, C. N., M. Grosell, J. N. Lott, and C. M. Wood. 2001. Copper metabolism and gut morphology in rainbow trout (*Oncorhynchus mykiss*) during chronic sublethal dietary copper exposure. Canadian Journal of Fisheries and Aquatic Sciences **58**:293-305.

Kang, Y.-J., and M. D. Eenger. 1987. Effect of cellular glutathione depletion on cadmium-induced cytotoxicity in human lung carcinoma cells. Cell biology and toxicology **3**:347-360.

Kapustka, L. A. 2004. Issue paper on the ecological effects of metals. US Environmental Protection Agency.

Karakoc, M., and S. Dincer. 2003. Effect of temperatures on zinc accumulation in the gill, liver, and kidney of *Oreochromis niloticus* (L. 1758). Bulletin of environmental contamination and toxicology **71**:1077-1083.

Kargın, F., and H. Çoğun. 1999. Metal interactions during accumulation and elimination of zinc and cadmium in tissues of the freshwater fish *Tilapia nilotica*. Bulletin of environmental contamination and toxicology **63**:511-519.

Kaur, K., and K. Bajwa. 1987. Effect of zinc and cadmium on early life stages of common carp, *Cyprinus carpio* Linn. Annals of Biology **3**:28-33.

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

Kim, S.-G., J.-H. Jee, and J.-C. Kang. 2004. Cadmium accumulation and elimination in tissues of juvenile olive flounder, Paralichthys olivaceus after sub-chronic cadmium exposure. Environmental Pollution **127**:117-123.

Kim, S. D., M. B. Gu, H. E. Allen, and D. K. Cha. 2001. Physicochemical factors affecting the sensitivity of Ceriodaphnia dubia to copper. Environmental Monitoring and Assessment **70**:105-116.

Kjoss, V. A., M. Grosell, and C. M. Wood. 2005. The influence of dietary Na on Cu accumulation in juvenile rainbow trout exposed to combined dietary and waterborne Cu in soft water. Archives of Environmental Contamination and Toxicology **49**:520-527.

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

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

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

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

Kovarova, J., R. Kizek, V. Adam, D. Harustiakova, O. Celechovska, and Z. Svobodova. 2009. Effect of cadmium chloride on metallothionein levels in carp. Sensors **9**:4789-4803.

Krasne, S., G. Eisenman, and G. Szabo. 1971. Freezing and melting of lipid bilayers and the mode of action of nonactin, valinomycin, and gramicidin. Science **174**:412-415.

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

Kumari, M. R., M. Hiramatsu, and M. Ebadi. 1998. Free radical scavenging actions of metallothionein isoforms I and II. Free radical research **29**:93-101.

Kwok, M. L., Z. P. Li, T. Y. S. Law, and K. M. Chan. 2020. Promotion of cadmium uptake and cadmium-induced toxicity by the copper transporter CTR1 in HepG2 and ZFL cells. Toxicology reports **7**:1564-1570.

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

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

Lannig, G., J. F. Flores, and I. M. Sokolova. 2006. Temperature-dependent stress response in oysters, *Crassostrea virginica*: pollution reduces temperature tolerance in oysters. Aquatic Toxicology **79**:278-287.

Lanno, R., B. Hickie, and D. Dixon. 1989. Feeding and nutritional considerations in aquatic toxicology. Pages 525-531 Environmental Bioassay Techniques and their Application. Springer.

Larsson, Å., B. E. Bengtsson, and C. Haux. 1981. Disturbed ion balance in flounder, *Platichthys flesus* L. exposed to sublethal levels of cadmium. Aquatic Toxicology **1**:19-35.

Larsson, D. J., K. Kinnberg, J. Sturve, E. k. Stephensen, M. Skön, and L. Förlin. 2002. Studies of masculinization, detoxification, and oxidative stress responses in

guppies (*Poecilia reticulata*) exposed to effluent from a pulp mill. Ecotoxicology and Environmental Safety **52**:13-20.

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

Lavut, A., and D. Raveh. 2012. Sequestration of highly expressed mRNAs in cytoplasmic granules, P-bodies, and stress granules enhances cell viability. PLoS Genet **8**:e1002527.

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

Lee, S. R. 2018. Critical role of zinc as either an antioxidant or a prooxidant in cellular systems. Oxidative medicine and cellular longevity **2018**.

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

Li, J., R. A. C. Lock, P. H. M. Klaren, H. G. P. Swarts, F. M. A. H. S. Stekhoven, S. E. W. Bonga, and G. Flik. 1996. Kinetics of Cu2+ inhibition of Na+/K+-ATPase. Toxicology Letters **87**:31-38.

Li, J., E. Quabius, S. W. Bonga, G. Flik, and R. Lock. 1998. Effects of water-borne copper on branchial chloride cells and Na+/K+-ATPase activities in Mozambique tilapia (*Oreochromis mossambicus*). Aquatic Toxicology **43**:1-11.

Li, Z.-H., P. Li, and Y. Wu. 2020. Effects of waterborne mercury at different temperatures on hematology and energy metabolism in grass carp (*Ctenopharyngodon idella*). International Journal of Environmental Science and Technology:1-10.

Liem, K. F., W. E. Bemis, W. F. Walker, and L. Grande. 2001. Functional anatomy of the vertebrates: an evolutionary perspective.

Lin, H., and D. J. Randall. 1993. H+-Atpase Activity in Crude Homogenates of Fish Gill Tissue - Inhibitor Sensitivity and Environmental and Hormonal-Regulation. Journal of Experimental Biology **180**:163-174.

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

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. methods **25**:402-408.

Livingstone, D. 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Marine pollution bulletin **42**:656-666.

Livingstone, D. 2003. Oxidative stress in aquatic organisms in relation to pollution and aquaculture. Revue de Medecine Veterinaire **154**:427-430.

Lizardo-Daudt, H., and C. Kennedy. 2008. Effects of cadmium chloride on the development of rainbow trout *Oncorhynchus mykiss* early life stages. Journal of fish Biology **73**:702-718.

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

Long, Y., L. Li, Q. Li, X. He, and Z. Cui. 2012. Transcriptomic characterization of temperature stress responses in larval zebrafish. PloS one **7**:e37209.

Loro, V. L., L. Nogueira, S. R. Nadella, and C. M. Wood. 2014. Zinc bioaccumulation and ionoregulatory impacts in Fundulus heteroclitus exposed to sublethal waterborne zinc at different salinities. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **166**:96-104.

Luoma, S., and P. Rainbow. 2008. Sources and cycles of trace metals. Metal contamination in aquatic environments: science and lateral management. Cambridge University Press, Cambridge **66**.

Luoma, S. N. 1983. Bioavailability of trace metals to aquatic organisms—a review. Science of The Total Environment **28**:1-22.

Lushchak, V. I. 2011. Environmentally induced oxidative stress in aquatic animals. Aquatic Toxicology **101**:13-30.

Lutsenko, S. 2010. Human copper homeostasis: a network of interconnected pathways. Current opinion in chemical biology **14**:211-217.

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

Maret, W. 2019. The redox biology of redox-inert zinc ions. Free Radical Biology and Medicine **134**:311-326.

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

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

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

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

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

McGeachy, S., and D. Dixon. 1989. The impact of temperature on the acute toxicity of arsenate and arsenite to rainbow trout (*Salmo gairdneri*). Ecotoxicology and Environmental Safety **17**:86-93.

McGeer, J. C., S. Nadella, D. H. Alsop, L. Hollis, L. N. Taylor, D. G. McDonald, and C. M. Wood. 2007. Influence of acclimation and cross-acclimation of metals on acute Cd toxicity and Cd uptake and distribution in rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology **84**:190-197.

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

McGeer, J. C., C. Szebedinszky, D. G. McDonald, and C. M. Wood. 2000a. Effects of chronic sublethal exposure to waterborne Cu, Cd or Zn in rainbow trout 2: tissue specific metal accumulation. Aquatic Toxicology **50**:245-256.

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

McRae, N. K., S. Gaw, and C. N. Glover. 2016. Mechanisms of zinc toxicity in the galaxiid fish, *Galaxias maculatus*. Comparative Biochemistry Physiology **179**:184-190.

Meister, A., and M. E. Anderson. 1983. Glutathione. Annual review of biochemistry **52**:711-760.

Metz, J. R., E. H. Van Den Burg, S. E. W. Bonga, and G. Flik. 2003. Regulation of branchial Na+/K+-ATPase in common carp *Cyprinus carpio* L. acclimated to different temperatures. Journal of Experimental Biology **206**:2273-2280.

Michiels, E. D., L. Vergauwen, A. Hagenaars, E. Fransen, S. V. Dongen, S. J. Van Cruchten, L. Bervoets, and D. Knapen. 2017. Evaluating complex mixtures in the zebrafish embryo by reconstituting field water samples: A metal pollution case study. International journal of molecular sciences **18**:539.

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

Minghetti, M., M. J. Leaver, and S. G. George. 2010. Multiple Cu-ATPase genes are differentially expressed and transcriptionally regulated by Cu exposure in sea bream, *Sparus aurata*. Aquatic Toxicology **97**:23-33.

Monteiro, S. M., J. M. Mancera, A. Fontainhas-Fernandes, and M. Sousa. 2005. Copper induced alterations of biochemical parameters in the gill and plasma of *Oreochromis niloticus*. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology **141**:375-383.

Muto, N., H.-W. Ren, G.-S. Hwang, S. Tominaga, N. Itoh, and K. Tanaka. 1999. Induction of two major isoforms of metallothionein in crucian carp (*Carassius cuvieri*) by air-pumping stress, dexamethasone, and metals. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology **122**:75-82.

Nichols, J., and R. Playle. 2004. Influence of temperature on silver accumulation and depuration in rainbow trout. Journal of fish Biology **64**:1638-1654.

Nicorelli, E., M. Gerdol, F. Buonocore, A. Pallavicini, G. Scapigliati, L. Guidolin, P. Irato, F. Corrà, and G. Santovito. 2018. First evidence of T cell restricted intracellular antigen (TIA) protein gene expression in Antarctic fish. Pages 127-127 *in* ISJ-invertebrate survival journal.

Nilsen, T. O., L. O. Ebbesson, O. G. Kverneland, F. Kroglund, B. Finstad, and S. O. Stefansson. 2010. Effects of acidic water and aluminum exposure on gill Na+, K+-ATPase α-subunit isoforms, enzyme activity, physiology and return rates in Atlantic salmon (*Salmo salar* L.). Aquatic Toxicology **97**:250-259.

Niyogi, S., C. Kamunde, and C. Wood. 2006. Food selection, growth and physiology in relation to dietary sodium chloride content in rainbow trout (*Oncorhynchus mykiss*) under chronic waterborne Cu exposure. Aquatic Toxicology **77**:210-221.

Niyogi, S., R. Kent, and C. M. Wood. 2008. Effects of water chemistry variables on gill binding and acute toxicity of cadmium in rainbow trout (*Oncorhynchus mykiss*): A biotic ligand model (BLM) approach. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **148**:305-314.

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

Niyogi, S., G. G. Pyle, and C. M. Wood. 2007. Branchial versus intestinal zinc uptake in wild yellow perch (*Perca flavescens*) from reference and metal-contaminated aquatic ecosystems. Canadian Journal of Fisheries and Aquatic Sciences **64**:1605-1613.

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

Niyogi, S., and C. M. Wood. 2003. Effects of chronic waterborne and dietary metal exposures on gill metal-binding: implications for the biotic ligand model. Human and Ecological Risk Assessment **9**:813-846.

Nordberg, M. 1998. Metallothioneins: historical review and state of knowledge. Talanta **46**:243-254.

Norwood, W., U. Borgmann, D. Dixon, and A. Wallace. 2003. Effects of metal mixtures on aquatic biota: a review of observations and methods. Human and Ecological Risk Assessment **9**:795-811.

Ochi, T., F. Otsuka, K. Takahashi, and M. Ohsawa. 1988. Glutathione and metallothioneins as cellular defense against cadmium toxicity in cultured Chinese hamster cells. Chemico-biological interactions **65**:1-14.

Ohlberger, J., G. Staaks, T. Petzoldt, T. Mehner, and F. Hölker. 2008. Physiological specialization by thermal adaptation drives ecological divergence in a sympatric fish species pair. Evolutionary Ecology Research **10**:1173-1185.

Olsson, P.-E., P. Kling, and C. Hogstrand. 1998. Mechanisms of heavy metal accumulation and toxicity in fish. Pages 321-350 Metal metabolism in aquatic environments. Springer.

Olszewska, M., J. J. Bujarski, and M. Kurpisz. 2012. P-bodies and their functions during mRNA cell cycle: Mini-review. Cell biochemistry and function **30**:177-182.

Osburn, W. O., and T. W. Kensler. 2008. Nrf2 signaling: an adaptive response pathway for protection against environmental toxic insults. Mutation Research/Reviews in Mutation Research **659**:31-39.

Pagenkopf, G. K. 1983. Gill surface interaction model for trace-metal toxicity to fishes: role of complexation, pH, and water hardness. Environmental science & technology **17**:342-347.

Pan, J., J. A. Plant, N. Voulvoulis, C. J. Oates, and C. Ihlenfeld. 2010. Cadmium levels in Europe: implications for human health. Environmental geochemistry and health **32**:1-12.

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

Pelgrom, S., L. Lamers, R. Lock, P. Balm, and S. W. Bonga. 1995. Interactions between copper and cadmium modify metal organ distribution in mature tilapia, *Oreochromis mossambicus*. Environmental Pollution **90**:415-423.

Pellegrini, M., and C. T. Baldari. 2009. Apoptosis and oxidative stress-related diseases: the p66Shc connection. Current molecular medicine **9**:392-398.

Peña-Llopis, S., M. D. Ferrando, and J. B. Peña. 2003. Fish tolerance to organophosphate-induced oxidative stress is dependent on the glutathione metabolism and enhanced by N-acetylcysteine. Aquatic Toxicology **65**:337-360.

Pena, M. M., J. Lee, and D. J. Thiele. 1999. A delicate balance: homeostatic control of copper uptake and distribution. The Journal of nutrition **129**:1251-1260.

Pereira, C. M., D. Deruytter, R. Blust, and K. A. De Schamphelaere. 2017. Effect of temperature on chronic toxicity of copper, zinc, and nickel to *Daphnia magna*. Environmental Toxicology and Chemistry **36**:1909-1916.

Pereira, T. C. B., M. M. Campos, and M. R. Bogo. 2016. Copper toxicology, oxidative stress and inflammation using zebrafish as experimental model. Journal of Applied Toxicology **36**:876-885.

Perera, P., S. Kodithuwakku, T. Sundarabarathy, and U. Edirisinghe. 2015. Bioaccumulation of cadmium in freshwater fish: an environmental perspective. Insight Ecol **4**:1-12.

Perry, S. F. 1997. The chloride cell: structure and function in the gills of freshwater fishes. Annual Review of Physiology **59**:325-347.

Perry, S. F., M. L. Beyers, and D. A. Johnson. 2000. Cloning and molecular characterisation of the trout (*Oncorhynchus mykiss*) vacuolar H (+)-ATPase B subunit. Journal of Experimental Biology **203**:459-470.

Pflugmacher, S. 2004. Promotion of oxidative stress in the aquatic macrophyte *Ceratophyllum demersum* during biotransformation of the cyanobacterial toxin microcystin-LR. Aquatic Toxicology **70**:169-178.

Pilehvar, A., R. M. Town, and R. Blust. 2019. The effect of thermal pre-incubation and exposure on sensitivity of zebrafish (*Danio rerio*) to copper and cadmium single and binary exposures. Aquatic Toxicology **213**:105226.

Pilehvar, A., R. M. Town, and R. Blust. 2020. The effect of copper on behaviour, memory, and associative learning ability of zebrafish (*Danio rerio*). Ecotoxicology and Environmental Safety **188**:109900.

Pillet, M., G. Castaldo, S. De Weggheleire, L. Bervoets, R. Blust, and G. De Boeck. 2019. Limited oxidative stress in common carp (*Cyprinus carpio*, L., 1758) exposed to a sublethal tertiary (Cu, Cd and Zn) metal mixture. Comparative Biochemistry and Physiology **218**:70-80.

Pillet, M., G. Castaldo, E. Rodgers, V. Poleksić, B. Rašković, L. Bervoets, R. Blust, and G. De Boeck. 2020. Physiological performance of common carp (Cyprinus carpio, L., 1758) exposed to a sublethal copper/zinc/cadmium mixture. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **242**:108954.

Pillet, M., G. Castaldo, E. Rodgers, V. Poleksić, B. Rašković, L. Bervoets, R. Blust, and G. De Boeck. 2021. Physiological performance of common carp (*Cyprinus carpio*, L., 1758) exposed to a sublethal copper/zinc/cadmium mixture. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **242**:108954.

Pinto, E., T. C. Sigaud-kutner, M. A. Leitao, O. K. Okamoto, D. Morse, and P. Colepicolo. 2003. Heavy metal–induced oxidative stress in algae 1. Journal of phycology **39**:1008-1018.

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

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

Pourang, N. 1995. Heavy metal bioaccumulation in different tissues of two fish species with regards to their feeding habits and trophic levels. Environmental Monitoring and Assessment **35**:207-219.

Prasad, A. S., and B. Bao. 2019. Molecular mechanisms of zinc as a pro-antioxidant mediator: clinical therapeutic implications. Antioxidants **8**:164.

Preston, S., N. Coad, J. Townend, K. Killham, and G. I. Paton. 2000. Biosensing the acute toxicity of metal interactions: are they additive, synergistic, or antagonistic? Environmental Toxicology and Chemistry: An International Journal **19**:775-780.

Pretto, A., V. L. Loro, V. M. Morsch, B. S. Moraes, C. Menezes, B. Clasen, L. Hoehne, and V. Dressler. 2010. Acetylcholinesterase activity, lipid peroxidation, and

bioaccumulation in silver catfish (*Rhamdia quelen*) exposed to cadmium. Archives of Environmental Contamination and Toxicology **58**:1008-1014.

Pyle, G., S. Swanson, and D. Lehmkuhl. 2002. The influence of water hardness, pH, and suspended solids on nickel toxicity to larval fathead minnows (*Pimephales promelas*). Water, Air, and Soil Pollution **133**:215-226.

Radhakrishnaiah, K., A. Suresh, and B. Sivaramakrishna. 1993. Effect of sublethal concentration of mercury and zinc on the energetics of a freshwater fish *Cyprinus carpio* (Linnaeus). Acta biologica Hungarica **44**:375-385.

Rainbow, P., C. Amiard-Triquet, J. Amiard, B. Smith, and W. Langston. 2000. Observations on the interaction of zinc and cadmium uptake rates in crustaceans (amphipods and crabs) from coastal sites in UK and France differentially enriched with trace metals. Aquatic Toxicology **50**:189-204.

Rainbow, P. S. 2007. Trace metal bioaccumulation: models, metabolic availability and toxicity. Environment international **33**:576-582.

Rajeshkumar, S., Y. Liu, J. Ma, H. Y. Duan, and X. Li. 2017. Effects of exposure to multiple heavy metals on biochemical and histopathological alterations in common carp, *Cyprinus carpio* L. Fish & Shellfish Immunology **70**:461-472.

Ren, H.-W., N. Itoh, M. Kanekiyo, S. TOMINAGA, J. KOHROKI, G.-S. HWANG, T. NAKANISHI, N. MUTO, and K. TANAKA. 2000. Two metallothioneins in the fresh-water fish, crucian carp (*Carassius cuvieri*): cDNA cloning and assignment of their expression isoforms. Biological and Pharmaceutical Bulletin **23**:145-148.

Reynders, H., L. Bervoets, M. Gelders, W. De Coen, and R. Blust. 2008. Accumulation and effects of metals in caged carp and resident roach along a metal pollution gradient. Science of The Total Environment **391**:82-95.

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

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

Rhee, J.-S., S. Raisuddin, D.-S. Hwang, K.-W. Lee, I.-C. Kim, and J.-S. Lee. 2009. Differential expression of metallothionein (MT) gene by trace metals and endocrine-disrupting chemicals in the hermaphroditic mangrove killifish, *Kryptolebias marmoratus*. Ecotoxicology and Environmental Safety **72**:206-212.

Roesijadi, G. 1992. Metallothioneins in metal regulation and toxicity in aquatic animals. Aquatic Toxicology **22**:81-113.

Roesijadi, G. 1996. Metallothionein and its role in toxic metal regulation. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology **113**:117-123.

Rogers, J. T., M. Patel, K. M. Gilmour, and C. M. Wood. 2005. Mechanisms behind Pb-induced disruption of Na+ and Cl− balance in rainbow trout (*Oncorhynchus mykiss*). American Journal of Physiology-Regulatory, Integrative and Comparative Physiology **289**:R463-R472.

Rogers, J. T., and C. M. Wood. 2004. Characterization of branchial lead-calcium interaction in the freshwater rainbow trout *Oncorhynchus mykiss*. Journal of Experimental Biology **207**:813-825.

Rombough, P. J., and E. Garside. 1984. Disturbed ion balance in alevins of Atlantic salmon *Salmo salar* chronically exposed to sublethal concentrations of cadmium. Canadian Journal of Zoology **62**:1443-1450.

Saibu, Y., A. Jamwal, R. Feng, D. Peak, and S. Niyogi. 2018. Distribution and speciation of zinc in the gills of rainbow trout (*Oncorhynchus mykiss*) during acute waterborne zinc exposure: Interactions with cadmium or copper. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **206**:23-31.

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

Santovito, G., E. Piccinni, and P. Irato. 2008. An Improved method for rapid determination of the reduced and oxidized states of metallothioneins in biological samples. Nova Science Publishers Inc.: New York, NY, USA:101-124.

Sappal, R., M. Fast, D. Stevens, F. Kibenge, A. Siah, and C. Kamunde. 2015. Effects of copper, hypoxia and acute temperature shifts on mitochondrial oxidation in rainbow trout (*Oncorhynchus mykiss*) acclimated to warm temperature. Aquatic Toxicology **169**:46-57.

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

Sato, M., and I. Bremner. 1993. Oxygen free radicals and metallothionein. Free Radical Biology and Medicine **14**:325-337.

Schisa, J. A. 2012. New insights into the regulation of RNP granule assembly in oocytes. International review of cell and molecular biology **295**:233-289.

Schoenaers, S., L. Vergauwen, A. Hagenaars, L. Vanhaecke, H. AbdElgawad, H. Asard, A. Covaci, L. Bervoets, and D. Knapen. 2016. Prioritization of contaminated watercourses using an integrated biomarker approach in caged carp. Water research **99**:129-139.

Schwarzenbach, R. P., B. I. Escher, K. Fenner, T. B. Hofstetter, C. A. Johnson, U. Von Gunten, and B. Wehrli. 2006. The challenge of micropollutants in aquatic systems. Science **313**:1072-1077.

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

Shahsavarani, A., B. McNeill, F. Galvez, C. M. Wood, G. G. Goss, P. P. Hwang, and S. F. Perry. 2006. Characterization of a branchial epithelial calcium channel (ECaC) in freshwater rainbow trout (*Oncorhynchus mykiss*). Journal of Experimental Biology **209**:1928-1943.

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

Shekh, K., S. Tang, M. Hecker, and S. Niyogi. 2018. Investigating the role of ionoregulatory processes in the species-and life-stage-specific differences in sensitivity of rainbow trout and white sturgeon to cadmium. Environmental science & technology **52**:12868-12876.

Shekh, K., S. Tang, V. Kodzhahinchev, S. Niyogi, and M. Hecker. 2019. Species and life-stage specific differences in cadmium accumulation and cadmium induced oxidative stress, metallothionein and heat shock protein responses in white sturgeon and rainbow trout. Science of The Total Environment **673**:318-326.

Shrivastava, J., A. K. Sinha, S. Cannaerts, R. Blust, and G. De Boeck. 2017. Temporal assessment of metabolic rate, ammonia dynamics and ion-status in common carp during fasting: a promising approach for optimizing fasting episode prior to fish transportation. Aquaculture **481**:218-228.

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

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

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

Smith, N., F. Eddy, and C. Talbot. 1995. Effect of dietary salt load on transepithelial Na+ exchange in freshwater rainbow trout (*Oncorhynchus mykiss*). Journal of Experimental Biology **198**:2359-2364.

Sokolova, I. M., and G. Lannig. 2008. Interactive effects of metal pollution and temperature on metabolism in aquatic ectotherms: implications of global climate change. Climate Research **37**:181-201.

Sprague, J. 1971. Measurement of pollutant toxicity to fish—III: Sublethal effects and "safe" concentrations. Water research **5**:245-266.

Spry, D., P. Hodson, and C. Wood. 1988. Relative contributions of dietary and waterborne zinc in the rainbow trout, *Salmo gairdneri*. Canadian Journal of Fisheries and Aquatic Sciences **45**:32-41.

Spry, D. J., and C. M. Wood. 1985. Ion flux rates, acid–base status, and blood gases in rainbow trout, *Salmo gairdneri*, exposed to toxic zinc in natural soft water. Canadian Journal of Fisheries and Aquatic Sciences **42**:1332-1341.

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

Stohs, S. J., and D. Bagchi. 1995. Oxidative mechanisms in the toxicity of metal ions. Free Radical Biology and Medicine **18**:321-336.

Strižak, Ž., D. Ivanković, D. Pröfrock, H. Helmholz, A.-M. Cindrić, M. Erk, and A. Prange. 2014. Characterization of the cytosolic distribution of priority pollutant metals and metalloids in the digestive gland cytosol of marine mussels: seasonal and spatial variability. Science of The Total Environment **470**:159-170.

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

Szebedinszky, C., J. C. McGeer, D. G. McDonald, and C. M. Wood. 2001. Effects of chronic Cd exposure via the diet or water on internal organ-specific distribution and subsequent gill Cd uptake kinetics in juvenile rainbow trout (*Oncorhynchus mykiss*). Environmental Toxicology and Chemistry: An International Journal **20**:597-607.

Tao, S., T. Liang, J. Cao, R. Dawson, and C. Liu. 1999. Synergistic effect of copper and lead uptake by fish. Ecotoxicology and Environmental Safety **44**:190-195.

Taylor, E., M. Beaumont, P. Butler, J. Mair, and M. Mujallid. 1996. Lethal and sub-lethal effects of copper upon fish: a role for ammonia toxicity? Pages 85-114 *in* SEMINAR SERIES-SOCIETY FOR EXPERIMENTAL BIOLOGY. Cambridge University Press.

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

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

Thornalley, P. J., and M. Vašák. 1985. Possible role for metallothionein in protection against radiation-induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals. Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology **827**:36-44.

Thornton, I. 1995. Metals in the global environment: facts and misconceptions. International Council on Metals and the Environment.

Tolomeo, A., A. Carraro, R. Bakiu, S. Toppo, F. Garofalo, D. Pellegrino, M. Gerdol, D. Ferro, S. Place, and G. Santovito. 2019. Molecular characterization of novel mitochondrial peroxiredoxins from the Antarctic emerald rockcod and their

gene expression in response to environmental warming. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **225**:108580.

Tolomeo, A., A. Carraro, R. Bakiu, S. Toppo, S. Place, D. Ferro, and G. Santovito. 2016. Peroxiredoxin 6 from the Antarctic emerald rockcod: molecular characterization of its response to warming. Journal of Comparative Physiology B **186**:59-71.

Tripathi, B., S. Mehta, A. Amar, and J. Gaur. 2006. Oxidative stress in Scenedesmus sp. during short-and long-term exposure to Cu2+ and Zn2+. Chemosphere **62**:538-544.

Tunçsoy, M., and C. Erdem. 2014. Accumulation of copper, zinc and cadmium in liver, gill and muscle tissues of *Oreochromis niloticus* exposed to these metals separately and in mixture. Fresenius Environmental Bulletin **23**:1143-1149.

Ugedal, O., B. Jonsson, O. Njåstad, and R. Næumann. 1992. Effects of temperature and body size on radiocaesium retention in brown trout, *Salmo trutta*. Freshwater Biology **28**:165-171.

USEPA. 2007. Aquatic Life Ambient Freshwater Quality Criteria – Copper. EPA-822-R-07-001, 2007 revision. Washington, DC: US Environmental Protection Agency.

Vallee, B., I. Bertini, and H. Gary. 1986. A synopsis of zinc biology and pathology in zinc enzymes. Birkhauser, Boston.

Van Campenhout, K., H. G. Infante, P. T. Hoff, L. Moens, G. Goemans, C. Belpaire, F. Adams, R. Blust, and L. Bervoets. 2010. Cytosolic distribution of Cd, Cu and Zn, and metallothionein levels in relation to physiological changes in gibel carp (*Carassius auratus gibelio*) from metal-impacted habitats. Ecotoxicology and Environmental Safety **73**:296-305.

Van Cleef-Toedt, K. A., L. A. Kaplan, and J. F. Crivello. 2001. Killifish metallothionein messenger RNA expression following temperature perturbation and cadmium exposure. Cell stress & chaperones **6**:351.

Van Ginneken, L., M. J. Chowdhury, and R. Blust. 1999. Bioavailability of cadmium and zinc to the common carp, *Cyprinus carpio*, in complexing environments: a test for the validity of the free ion activity model. Environmental Toxicology and Chemistry: An International Journal **18**:2295-2304.

Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome biology **3**:1-12.

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

Verbost, P., G. Flik, R. Lock, and S. W. Bonga. 1988. Cadmium inhibits plasma membrane calcium transport. The Journal of membrane biology **102**:97-104.

Verbost, P. M., G. Flik, R. Lock, and S. Wendelaar Bonga. 1987. Cadmium inhibition of Ca2+ uptake in rainbow trout gills. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology **253**:R216-R221.

Verbost, P. M., J. Vanrooij, G. Flik, R. A. C. Lock, and S. E. W. Bonga. 1989. The Movement of Cadmium through Fresh-Water Trout Branchial Epithelium and

Its Interference with Calcium-Transport. Journal of Experimental Biology **145**:185-197.

Vergani, L. 2009. Metallothioneins in aquatic organisms: fish, crustaceans, molluscs and echinoderms. Metal ions in life sciences **5**:199-238.

Vergauwen, L., D. Benoot, R. Blust, and D. Knapen. 2010. Long-term warm or cold acclimation elicits a specific transcriptional response and affects energy metabolism in zebrafish. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology **157**:149-157.

Vergauwen, L., A. Hagenaars, R. Blust, and D. Knapen. 2013. Temperature dependence of long-term cadmium toxicity in the zebrafish is not explained by liver oxidative stress: evidence from transcript expression to physiology. Aquatic Toxicology **126**:52-62.

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

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

VLAREM II. 2010. Vlaams Reglement betreffende de Milieuvergunning, bijlage 2.3.1. Basismilieukwaliteitsnormen voor oppervlaktewater.

VMM. 2016. http://geoloket.vmm.be/Geoviews/.

Voss, B. 1985. Effects of temperature on the activity of pyruvate dehydrogenase in liver mitochondria of the rainbow trout (*Salmo gairdneri*). Journal of thermal biology **10**:131-135.

Waalkes, M. P., M. J. Harvey, and C. D. Klaassen. 1984. Relative in vitro affinity of hepatic metallothionein for metals. Toxicology Letters **20**:33-39.

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

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

Wang, Y. D., J. Fang, S. S. Leonard, and K. M. K. Rao. 2004. Cadmium inhibits the electron transfer chain and induces reactive oxygen species. Free Radical Biology and Medicine **36**:1434-1443.

Ward, A., E. Hensor, M. Webster, and P. Hart. 2010. Behavioural thermoregulation in two freshwater fish species. Journal of fish Biology **76**:2287-2298.

Watanabe, T., V. Kiron, and S. Satoh. 1997. Trace minerals in fish nutrition. Aquaculture **151**:185-207.

Wätjen, W., and D. Beyersmann. 2004. Cadmium-induced apoptosis in C6 glioma cells: influence of oxidative stress. Biometals **17**:65-78.

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

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

Willmer, P., G. Stone, and I. Johnston. 2009. Environmental physiology of animals. John Wiley & Sons.

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

Wilson, R., and E. Taylor. 1993. The physiological responses of freshwater rainbow trout, *Oncorhynchus mykiss*, during acutely lethal copper exposure. Journal of Comparative Physiology B **163**:38-47.

Wimmer, U., Y. Wang, O. Georgiev, and W. Schaffner. 2005. Two major branches of anti-cadmium defense in the mouse: MTF-1/metallothioneins and glutathione. Nucleic acids research **33**:5715-5727.

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

Witeska, M., B. Jezierska, and J. Chaber. 1995. The influence of cadmium on common carp embryos and larvae. Aquaculture **129**:129-132.

Woo, S., S. Yum, J. H. Jung, W. J. Shim, C.-H. Lee, and T.-K. Lee. 2006. Heavy metal-induced differential gene expression of metallothionein in Javanese medaka, *Oryzias javanicus*. Marine Biotechnology **8**:654-662.

Wood, C. M. 2011. An introduction to metals in fish physiology and toxicology: basic principles. Pages 1-51 Fish physiology. Elsevier.

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

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

Yang, H.-N., and H.-C. Chen. 1996. Uptake and elimination of cadmium by Japanese eel, *Anguilla japonica*, at various temperatures. Bulletin of environmental contamination and toxicology **56**:670-676.

Zebral, Y. D., M. Roza, J. da Silva Fonseca, P. G. Costa, C. S. de Oliveira, T. G. Zocke, J. L. Dal Pizzol, R. B. Robaldo, and A. Bianchini. 2019. Waterborne copper is more toxic to the killifish *Poecilia vivipara* in elevated temperatures: Linking oxidative stress in the liver with reduced organismal thermal performance. Aquatic Toxicology **209**:142-149.

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

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

Zheng, D., G. P. Feeney, R. D. Handy, C. Hogstrand, and P. Kille. 2014. Uptake epithelia behave in a cell-centric and not systems homeostatic manner in response to zinc depletion and supplementation. Metallomics **6**:154-165.

Zheng, D., G. P. Feeney, P. Kille, and C. Hogstrand. 2008. Regulation of ZIP and ZnT zinc transporters in zebrafish gill: zinc repression of ZIP10 transcription by an intronic MRE cluster. Physiological genomics **34**:205-214.

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

Zhou, Q., J. Zhang, J. Fu, J. Shi, and G. Jiang. 2008. Biomonitoring: an appealing tool for assessment of metal pollution in the aquatic ecosystem. Analytica chimica acta **606**:135-150.

Zhu, Q.-H., Z.-K. Zhou, D.-D. Tu, Y.-L. Zhou, C. Wang, Z.-P. Liu, W.-B. Gu, Y.-Y. Chen, and M.-A. Shu. 2018a. Effect of cadmium exposure on hepatopancreas and gills of the estuary mud crab (*Scylla paramamosain*): Histopathological changes and expression characterization of stress response genes. Aquatic Toxicology **195**:1-7.

Zhu, Q.-L., W.-Y. Li, and J.-L. Zheng. 2018b. Life-cycle exposure to cadmium induced compensatory responses towards oxidative stress in the liver of female zebrafish. Chemosphere **210**:949-957.

Curriculum Vitae

Personal information

Name:	Giovanni Castaldo
E-mail:	Giovanni.Castaldo@uantwerpen.be giovannicastaldo90@virgilio.it
Place and date of birth:	Maddaloni (CE), Italy, 08 November 1990

Education

2017 - Present	PhD researcher at the Systemic Physiological & Ecotoxicological Research (SPHERE) group University of Antwerp: "Mixed metal and temperature stress in aquatic environments: establishing functional links across different levels of organisation."
2013 - 2015	MSc in Marine Biology at Università Politecnica delle Marche, Ancona, Italy
2009 - 2013	BSc in Biological Sciences at Seconda Università degli Studi di Napoli, Caserta, Italy

Publications

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

Castaldo, G., Flipkens, G., Pillet, M., Town, R.M., Bervoets, L., Blust, R. and De Boeck, G., 2020. Antagonistic bioaccumulation of waterborne Cu (II) and Cd (II) in common carp (*Cyprinus carpio*) and effects on ion-homeostasis and defensive mechanisms. *Aquatic Toxicology*, *226*, p.105561.

Castaldo, G., Delahaut, V., Slootmaekers, B., Bervoets, L., Town, R.M., Blust, R. and De Boeck, G., 2020. 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*.

Castaldo, G., Pillet, M., Ameryckx, L., Bervoets, L., Town, R.M., Blust, R. and De Boeck, G., 2021. Temperature effects during a sublethal chronic metal mixture exposure on common carp (*Cyprinus carpio)*. *Frontiers in physiology*, *12*, p.284.

Castaldo, G., Nguyễn, T., Town, R.M., Bervoets, L., Blust, R. and De Boeck, G., 2021. Common carp exposed to binary mixtures of Cd (II) and Zn (II): a study on metal bioaccumulation and ion-homeostasis. Aquatic Toxicology, p.105875.

Conferences

Castaldo G., Blust R., De Boeck G.. Effects of heavy metal mixtures on bioaccumulation and defence mechanisms in common carp, *Cyprinus carpio*. Setac Rome 2018. Poster Presentation.

Castaldo G., Pillet M., Bervoets L., Blust R., De Boeck G. Effects of a sublethal metal mixture of 10%LC_{50} Cu, Zn and Cd on metal accumulation and ionoregulation in *Cyprinus carpio*. Setac Helsinki 2019. Platform presentation.

Castaldo G., Pillet M., Slootmaekers B., Bervoets L., Blust R., De Boeck G. Effects of metal mixture and temperature stress on metal accumulation and ionoregulation in *Cyprinus carpio*. SETAC online conference 2020 (SciCon). Platform presentation.