Comparative analysis of metal toxicity responses in aquatic invertebrate and vertebrate model organisms

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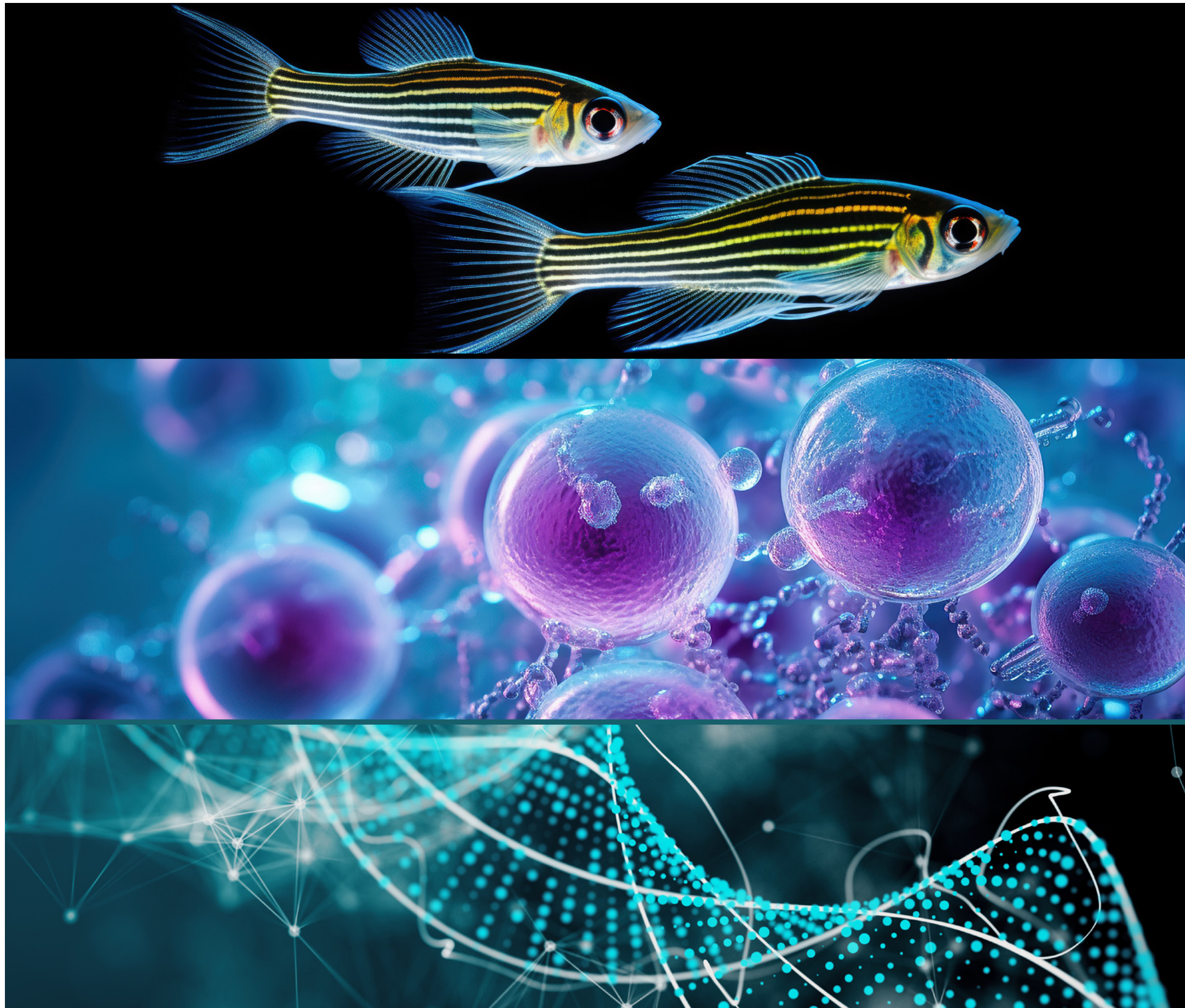

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Summary

Summary

The presence of metals in the environment has often been associated with various adverse effects on living systems, including humans. Given their ubiquitous nature, metals are omnipresent and often occur as mixtures and have therefore always been of concern. Despite the considerable attention given to this issue, there remains a lack of sufficient knowledge of the mechanisms underlying toxicological outcomes specific to each metal and/or each mixture. In addition, knowledge of the toxicity of metal mixtures in taxonomically distinct species is inadequately explored. Addressing these key questions is important for gaining a better understanding of the magnitude and mechanisms of toxicity in biological systems. In this context, our study aims to explore how exposures in a single and binary metal pollution scenario differentially affect aquatic vertebrate and invertebrate organisms across various biological levels. The study focuses on copper (Cu) and cadmium (Cd) as metal toxicants in three animal models: the zebrafish *(Danio rerio),* the water flea *(Daphnia magna)* and the planarian flatworm *(Schmidtea mediterranea).*

In the first chapter of this thesis, experiments were performed on the fertilised embryos and adults of zebrafish *(Danio rerio).* Both, embryos, and adults were exposed to sublethal levels of Cu and Cd as single and binary mixtures. Developmental, morphological, and functional endpoints were assessed in embryos at 96 hpf (hours post fertilisation). The results of this part of the work shows an increased sensitivity of both embryos and adults that were simultaneously exposed to both metals. Based on the transcriptional pattern of genes, the underlying mechanisms activated during the combined exposure were related to DNA damage and oxidative stress. In adults, the degree of toxicity and the underlying mode of action depended on the specific organ. Alterations in the activity of antioxidant genes were detected in all organs, but DNA damage was only detected in the gills under the given experimental conditions. Furthermore, the results indicate the inadequacy of relying solely on the extent of metal accumulation as a predictor of toxicity.

In the second chapter of this thesis, we conducted a comparative analysis of the individual and joint toxicity of Cu and Cd in the water flea *(Daphnia magna).* Our investigation focused on exploring the role of exposure pathways during the early and adult life stages. Animals were exposed to single and mixture of Cu and Cd through three distinct pathways: aqueous (dissolved in water), dietary (spiked with food) and their combination (water + food), over a

period of 7-days. The main objective of evaluating these exposure pathways, was to determine the relative importance of each in contributing to overall toxicity. Toxicity endpoints, including survival, growth, and reproduction were evaluated. The results of this part of the work show that the mixed exposure to Cu and Cd increased toxicity for both age groups of *Daphnia magna* compared to single exposures. However, neonates showed more sensitivity to stress, when exposed to similar metal concentrations compared to adults. In addition, the severity of stress varied between different exposure pathways and was higher when metal treatments occurred in a combined exposure scenario. Oxidative stress was found to be a common toxicity biomarker of Cu and Cd co-exposure in both neonates and adults exposed via combined pathways.

The aim of the third chapter of this thesis was to determine the potential developmental and physiological perturbations in regenerating and intact (adult) planarian flatworms *(Schmidtea mediterranea)*. Regenerating planarians were used as a proxy for developmental toxicity. Underlying cellular and molecular events were assessed following single and mixed exposures to Cu and Cd over 7 and/or 14 days. The mixed exposure resulted in pronounced lethal and non-lethal morphological changes, neuroregenerative impairments, altered behaviour, and a decrease in survival. The mixed exposure additionally impaired the recovery in regenerating animals, indicating the severity of toxicity. Oxidative stress appeared as an underlying mechanism for toxicity in all exposure conditions. The transcriptional pattern of DNA repair genes in adult animals indicated adverse effects on DNA. Overall, our results demonstrate that the toxic effects of the individual metals increase significantly in mixed exposure scenarios, and that developing organisms are more susceptible than adults.

Overall, our study contributes to a better understanding of the single and mixture effects of metal toxicity across different biological levels and emphasises the importance of an in-depth approach in assessing the risks associated with metal pollution. The findings presented in this work show a stronger action of Cu and Cd as a mixture in different aquatic organisms under controlled conditions. In particular, our study reveals important developmental, behavioural, and molecular changes with some effects more manifested in mixtures compared to single metal exposures. Furthermore, our study highlights that relying solely on metal accumulation levels as a reliable predictor of toxicity is insufficient; however, it can still serve as a valuable biomarker of exposure. The absence of a clear relationship

between metal concentrations in tissues and observed effects emphasises the influence of internal compartmentalisation and the intricate molecular defence mechanisms involved in damage control and repair processes.

Samenvatting

De aanwezigheid van metalen in het milieu is vaak in verband gebracht met verschillende schadelijke effecten op levende systemen, waaronder de mens. Gezien hun alomtegenwoordigheid wordt algemeen aangenomen dat metalen voorkomen als complexe mengsels en daarom zijn ze altijd een bron van zorg geweest. Ondanks de aanzienlijke aandacht die aan dit onderwerp is besteed, is er nog steeds onvoldoende kennis over de mechanismen die ten grondslag liggen aan toxicologische resultaten die specifiek zijn voor elk metaal en/of elk mengsel. Bovendien is de kennis over de toxiciteit van metaalmengsels in taxonomisch verschillende soorten onvoldoende onderzocht. Het beantwoorden van deze vragen is belangrijk om een beter inzicht te krijgen in de omvang en mechanismen van toxiciteit in biologische systemen. In deze context is onze studie erop gericht om te onderzoeken hoe blootstelling in een enkelvoudig en binair verontreinigingsscenario met metalen een differentieel effect heeft op gewervelde en ongewervelde waterorganismen op verschillende biologische niveaus. Het onderzoek richt zich op koper (Cu) en cadmium (Cd) als metaaltoxische stoffen in drie diermodellen: de zebravis (*Danio rerio*), de watervlo (*Daphnia magna*) en de platworm (*Schmidtea mediterranea*).

In het eerste hoofdstuk van dit proefschrift werden experimenten uitgevoerd op bevruchte embryo's en volwassen zebravissen (*Danio rerio*). Zowel embryo's als volwassen dieren werden blootgesteld aan subletale niveaus van Cu en Cd als enkelvoudige en binaire mengsels. Ontwikkelings-, morfologische en functionele eindpunten werden beoordeeld in embryo's op 96 hpf (uur na bevruchting). De resultaten van dit deel van het werk tonen een verhoogde gevoeligheid van zowel embryo's als adulten die gelijktijdig aan beide metalen werden blootgesteld. Gebaseerd op het transcriptiepatroon van genen, waren de onderliggende mechanismen die geactiveerd werden tijdens de gecombineerde blootstelling gerelateerd aan DNA-schade en oxidatieve stress. Bij adulten hing de mate van toxiciteit en het onderliggende werkingsmechanisme af van het specifieke orgaan. Veranderingen in de activiteit van antioxidantgenen werden vastgesteld in alle organen, maar DNA-schade werd in de gegeven scenario's alleen vastgesteld in de kieuwen. Bovendien suggereren de resultaten dat de mate van metaalaccumulatie geen betrouwbare indicator van toxiciteit is, wat de interne dynamiek van de compartimentering en hantering van metalen weerspiegelt als functie van de blootstellingsconcentratie en -tijd.

In het tweede hoofdstuk van dit proefschrift werd de individuele en gemengde toxiciteit van Cu en Cd vergeleken in de watervlo (*Daphnia magna*). De rol van blootstellingsroutes werd onderzocht in hun vroege en volwassen levensstadia. Adulte dieren en neonaten van *Daphnia magna* werden gedurende 7 dagen blootgesteld aan enkelvoudige en mengsels van Cu en Cd via drie blootstellingsroutes: waterig (opgelost in water), via de voeding (vermengd met voedsel) en hun combinatie (water + voedsel). Het doel van de evaluatie van de blootstellingsroutes was om het relatieve belang van elke route voor toxiciteit te beoordelen. Eindpunten van toxiciteit, waaronder overleving, groei en voortplanting werden geëvalueerd. De resultaten van dit deel van het werk tonen aan dat de gemengde blootstelling van Cu en Cd de toxiciteit voor beide leeftijdsgroepen van *Daphnia magna* verhoogde in vergelijking met enkelvoudige blootstelling. Neonaten vertoonden echter een grotere gevoeligheid voor stress bij blootstelling aan vergelijkbare metaalconcentraties dan volwassenen. Bovendien varieerde de ernst van de stress tussen verschillende blootstellingsroutes en was deze hoger wanneer de metaalbehandelingen plaatsvonden in een gecombineerd blootstellingsscenario. Oxidatieve stress bleek een veel voorkomende biomerker te zijn voor de toxiciteit van gelijktijdige blootstelling aan Cu en Cd bij zowel neonaten als bij adulten die via gecombineerde blootstellingsroutes werden blootgesteld.

Het doel van het derde hoofdstuk van dit proefschrift was het bepalen van de potentiële ontwikkelings- en fysiologische verstoringen in regenererende en intacte (volwassen) planarian platwormen (*Schmidtea mediterranea*). Regenererende planarianen werden gebruikt als proxy voor ontwikkelingstoxiciteit. Onderliggende cellulaire en moleculaire gebeurtenissen werden beoordeeld na enkelvoudige en gemengde blootstelling aan Cu en Cd gedurende 7 en/of 14 dagen. De gemengde blootstelling resulteerde in uitgesproken letale en niet-letale morfologische veranderingen, neuroregeneratieve stoornissen, veranderd gedrag en een afname in overleving. De gemengde blootstelling verminderde bovendien het herstel bij regenererende dieren, wat de ernst van de toxiciteit aangeeft. Oxidatieve stress bleek een onderliggend mechanisme te zijn voor toxiciteit in alle blootstellingsomstandigheden. Het transcriptiepatroon van DNA-herstelgenen in volwassen dieren wees op nadelige effecten op het DNA. In het algemeen tonen onze resultaten aan dat de toxische effecten van de afzonderlijke metalen aanzienlijk toenemen in gemengde blootstellingsscenario's en dat organismen in ontwikkeling vatbaarder zijn dan volwassenen.

In het algemeen draagt onze studie bij aan een beter begrip van de interactieve effecten van metaaltoxiciteit op verschillende biologische niveaus en benadrukt deze het belang van een diepgaande benadering bij het beoordelen van de risico's van metaalverontreiniging. De bevindingen in dit proefschrift tonen een sterkere werking van Cu en Cd als mengsel in verschillende aquatische organismen onder gecontroleerde omstandigheden. In het bijzonder onthult onze studie belangrijke ontwikkelings-, gedrags- en moleculaire veranderingen, waarbij sommige effecten zich meer manifesteren bij mengsels dan bij blootstelling aan enkelvoudige metalen. Verder laat onze studie zien dat metaalaccumulatieniveaus alleen geen betrouwbare voorspeller zijn van toxiciteit; desondanks kunnen ze dienen als een waardevolle biomerker van blootstelling. Het ontbreken van een duidelijk verband tussen metaalconcentraties in weefsels en waargenomen effecten benadrukt de invloed van interne compartimentering en de ingewikkelde moleculaire verdedigingsmechanismen die betrokken zijn bij schadecontrole en herstelprocessen.

General Introduction

General Introduction

Despite the remarkable growth in technological advancement, concentrations of metals in the environment continue to exceed the limits recommended by regulatory agencies around the world (Sharma et al., 2021). The toxicity of metals such as arsenic, cadmium, copper, nickel, mercury, chromium, zinc, and lead are of great concern from both environmental and public health perspectives. In addition, the interactions between different metals as well as with other substances and various host factors are a subject of attention. The toxicity of these metals involves many mechanistic aspects, some of which are not clearly elucidated or understood. There is still insufficient knowledge about specific effects of individual metals and/or specific mixtures. Furthermore, knowledge of the developmental toxicity of specific metal mixtures in taxonomically distinct species is a less explored area. However, over the years it has become clear that each metal has unique characteristics and physicochemical properties that triggers specific toxicological mechanisms of action. Deciphering the molecular mechanisms of toxicity and the degree of similarity in the biological response pathways responsible for toxicity in different species especially in the context of mixed exposures, is important and can be used in risk assessment to improve our health and preserve our environment (Mumtaz and Pohl, 2012; Hernandez et al., 2019; Xia et al., 2020).

1. Metals - a continuous environmental problem

Toxic metals are one of the oldest environmental problems which continues till today with new dimensions. Since the last few decades, metal pollution has received immense attention due to their extensive use, discharge into the environment and toxicities associated with them (Herawati et al., 2000; He et al., 2005; Craig et al., 2007; Vardhan et al., 2019; Balali-Mood et al., 2021). At present, the demand for metals is increasing rapidly and this trend is expected to continue over the next decades (UNEP 2013; Takuma et al., 2021). The presence of metals in the environment affects both the ecological integrity and the well-being of different life forms. Unlike organic pollutants, metals are not biodegradable and tend to accumulate in living organisms. These can originate from multiple sources, including natural processes (rock weathering, forest fires, volcanic eruptions, etc) and human activities (industries, agriculture, wastewater, runoffs, mining and metallurgy) and end up in different environmental compartments (soil, water, air and their interface) (Koller and Saleh, 2018). A natural exposure to a metal may be harmless to humans and other species. However,

widespread contamination from industrialisation and other anthropogenic sources, which is the general background exposure in some countries, cause adverse health effects in sensitive populations.

Some metals are nutritionally essential at relative low concentrations (e.g., copper, iron, cobalt and zinc), due to their role in various biochemical processes, but can become toxic at elevated concentrations or in certain forms (Saad et al., 2014). Other metals are non-essential (e.g., cadmium, mercury, arsenic, lead) and potential sources of toxicity even at low concentrations. Although toxicity thresholds and modes of action may vary between essential and non-essential metal ions, they share some common toxicity effects, such as impairment of ion regulation (Moulis, 2010; Alsop and Wood, 2011) and induction of oxidative stress (Sevcikova et al., 2011; Wu et al., 2019). Factors influencing metal toxicity may be related to the form and innate chemical activity of metal ions, route of exposure, differences in metabolism due to differences in structural and functional organisation of organisms and biotic handling of metal ions. In the present study, copper (Cu) and cadmium (Cd) were used as toxicants to investigate the underlying molecular mechanisms and species-specific responses of these metals under single and co-exposure scenarios. These metals were chosen because their biological (Cu), toxicological relevance (Cu and Cd) and widespread presence in the environment at sometimes highly elevated concentrations.

1.1 Copper

1.1.1 Sources of copper in the environment

Copper (Cu) occurs naturally in many minerals, such as cuprite (Cu_2O), tenorite (CuO), malachite ($CuCO_3 \cdot Cu(OH)_2$), azurite ($2CuCO_3 \cdot Cu(OH)_2$), antlerite ($CuSO_4 \cdot 2Cu(OH)_2$), brochantite ($CuSO_4 \cdot 3Cu(OH)_2$), chrysocolla ($CuO \cdot SiO_2 \cdot 2H_2O$), chalcopyrite ($CuFeS_2$), chalcocite ($Cu_2S$), covellite ($CuS$), and bornite ($Cu_5FeS_4$). It also occurs uncombined as solely copper metal (ATSDR, 2022). Copper and its compounds are released into the environment through both natural processes and anthropogenic activities. The natural sources of Cu include volcanic eruptions, decaying vegetation, sea spray, wind-blown dust, and forest fires. Major anthropogenic activities that contribute to the continuous release of Cu into the environment include mining, metal production, chemical industries, and use of pesticides. For example, 'Bordeaux mixture' (copper sulfate and lime) is still used to control fungal attack on grapes and other crops (Lamichhane et al., 2018). Copper-based antifouling paints

have been used for several centuries to prevent fouling of boats and are still widely used. Cu in various forms (including copper naphthenate, chromated copper arsenate, copper chromo-fluoride, ammoniacal copper zinc arsenate, ammoniacal copper quat, and copper 8-hydroxyquinolinate) has long been used to protect wood from insects and rot. Cu-based algaecides are routinely used to control noxious algal blooms in freshwaters (Bishop et al., 2018). Drinking water can contribute significantly to daily copper intake due to the widespread use of copper in household plumbing system such as pipes, faucets, and water fixtures (Health Canada, 2019). Cu concentrates in water when it leaches from these plumbing materials.

1.1.2 Environmental levels and exposures to copper

When Cu and its compounds are released into water, the dissolved Cu can be transported in surface water either as a compound or in a free form, or more likely to become bound to suspended inorganic and organic particles or convert to other forms that can settle in sediments. This limits the exposure to Cu unless sediments are stirred up, such as by resuspension and ingestion of sediments. The distribution of Cu depends on pH, oxidation-reduction potential in the local environment, and the presence of competing metal ions and inorganic anions (Rader et al., 2019). The strong tendency of copper to bind to various dissolved ligands and solid phases has important implications for its fate, as such binding affects the transformations and fluxes of copper between different compartments in the aquatic environment (Rader et al., 2019).

Naturally occurring concentrations of Cu in freshwater systems have been reported to be in the range of 0.2 – 30 µg/L (0.003 – 0.47 µM) (USEPA 2007b; ATSDR, 2022). Anthropogenic input can increase the ambient Cu levels up to 100 µg/L (1.57 µM) or as high as 200 µg/L (3.14 µM) (USEPA 2007; Luzio et al., 2013). Copper is one of the metals most commonly found in surface waters at concentrations exceeding the environmental quality standards set by the European Union (Delahaut et al., 2020). As reported by Delahaut et al., (2020) the total concentrations of copper measured by the Flemish Environmental Agency ranged from 0.02–0.54 µM (1.27–34.32 µg/L) in 2014. Exceedances of these levels in both surface and drinking waters has been reported worldwide.

Cu is also carried into the air with metallurgical dust. Most reports have indicated <1 µg/L of Cu in open marine water, however higher levels can be measured in coastal regions and estuaries (Ellingsen et al., 2007). In the air Cu concentration ranges from a few nanograms to about 200 ng/m^3. Near smelters that process copper ore into metal, concentrations can reach 5,000 ng/m^3 (ATSDR, 2004). Indoors, it is primarily released by combustion processes. Copper occurs in sediments at concentrations ranging from 0.12 to 35,700 mg/kg (WQP 2020, ATSDR, 2022). EPA reports levels in soil of 0.58 to 334 mg/kg (WQP 2020). In 2007, USGS conducted a geochemical and mineralogical survey of soils of the conterminous United States. The mean concentration of Cu calculated from the 4841 samples taken was 17.9 mg/kg, with values ranging from <0.5 to 996 mg/kg (ATSDR, 2022). In addition, humans may be exposed to Cu through drinking water, which may contain Cu levels above the acceptable drinking water standard of 1.3mg/L, especially when Cu plumbing and brass water fixtures are used (ATSDR,2004; Health Canada, 2019).

1.1.3 Biological role of copper

Cu is nutritionally important at low levels and plays a key role in several biochemical processes. (Linder, 2001, De Romaña et al., 2011; Ruiz et al., 2021). It is a redox – active metal and in biological systems it largely exists in cupric form (Cu^{2+}). Cu is required for maintaining the strength of the skin, blood vessels, epithelial and connective tissues and plays a role in the production of haemoglobin, myelin, and melanin and ensures normal thyroid function the formation of melanin pigment and myelin covering around the neurons (Osredkar and Sustar, 2011; Gaetke et al., 2014). The ability of Cu to undergo redox cycling from the stable oxidised Cu^{2+} to unstable reduced Cu^{+} form makes it biologically significant (Stern, et. al., 2007; Kardos et al., 2018). It can act as both an antioxidant and a prooxidant. As an antioxidant, it acts as a co-factor for the functioning of many vital enzyme systems such as superoxide dismutase (SOD), ceruloplasmin, lysyl oxidase, mitochondrial cytochrome *c* oxidase and tyrosinase (Hefnawy and El-khaiat, 2015) and scavenges or neutralizes free radicals, hence reduces, or prevents some of the damage they cause. When Cu temporarily acts as a prooxidant, it promotes free radical damage which can lead to oxidative stress through enhanced formation of reactive oxygen species (ROS) via Fenton and Haber–Weiss reactions (Rensing and Grass, 2003; Collin 2019) (see Figure 1). Cu homeostasis ensures that Cu levels in the body are maintained without causing cellular damage. Disruption of Cu homeostasis has been associated with tissue damage and several

diseases (Bleackley and Macgillivray, 2011; de Romana et al., 2011; Hefnawy and El-Khaiat, 2015).

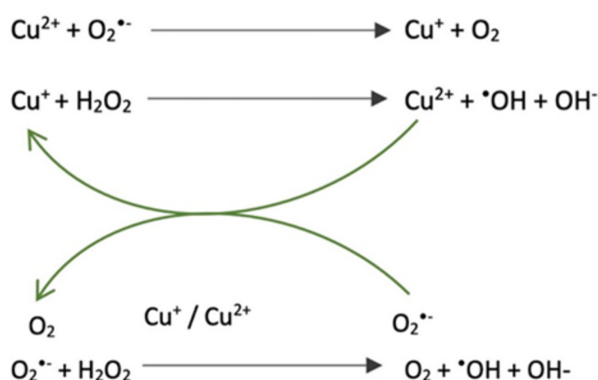

Figure 1. Fenton and Haber – Weiss reaction for Cu. Fenton and Haber – Weiss reactions are frequently responsible for the generation of reactive oxygen species (ROS) in living cells. In this reaction hydrogen peroxide (H_2O_2) is decomposed to hydroxyl radical (•OH) and hydroxyl anion (OH^-) with the participation of metal ions such as Fe, Cu, Zn and Al. The •OH radical produced can cause oxidative DNA damage and other effects (Snehkina et al., 2019).

1.1.4 Copper homeostasis

Organisms have evolved several systems to maintain Cu homeostasis within cells to ensure that essential functions are supported while minimizing toxicity risks (Gaetke et al., 2014). Copper uptake depends upon several factors, including its chemical form, interaction with other chemical components and physiological conditions (Johnson, 1989; Foder et al, 2020; Amundson et al., 2024). A higher intake of Cu can lead to increased absorption, while the chemical form and solubility influence its bioavailability (Wapnir, 1998). Although both vertebrates and invertebrates require Cu for essential cellular functions, the mechanisms for homeostasis can vary between these groups (Taylor & Anstiss, 1999; Foder et al., 2020). For example, some invertebrates, such as molluscs and some arthropods utilize hemocyanin, a copper-containing protein, for oxygen transport instead of haemoglobin present in mammals and many other species (Coates & Costa-Paiva, 2020). Additionally, the transporters and chaperone proteins involved in Cu homeostasis in invertebrates may exhibit structural and functional differences from those in vertebrates (Fodor et al., 2020).

In vertebrates, including mammals, Cu absorption is more complex, and primarily occurs in the gut. Upon absorption, Cu is transported to the liver, the major organ involved in copper

metabolism, and distributed to other tissues as needed (ATSDR, 2022). Any excess Cu is efficiently excreted via bile, to maintain Cu homeostasis in the body. The homeostasis of Cu is regulated through a complex system of Cu transporters and chaperone proteins, which are crucial for both the influx and detoxification of Cu (see Table. 1) (Evans and Halliwell, 1994; Burkitt, 2001; Prohaska, 2008). The influx of reduced Cu ions is regulated by two transmembrane solute carrier transporters, the high affinity copper uptake protein 1 (CTR1) and the low affinity CTR2. CTR1 is the major copper importer located at the apical site of enterocytes, but there are also reports showing a possible role of DMT1 in the transfer of Cu. CTR1 present at the plasma membrane is negatively regulated by the cellular Cu levels via a feedback mechanism (Chen et al., 2020). From the cell surface CTR1 undergoes internalization dependent degradation in response to elevated Cu levels (Molloy and Kaplan, 2009), which can be recycled back to the plasma membrane when extracellular Cu levels are reduced (Fukai et al., 2018). The regulation pattern and function of CTR2 are not well defined. Once inside the cell, Cu is coupled to chaperons and Cu-transporting ATPases expressed in tissues (Prohaska, 2008; Boal and Rosenweig, 2009). The antioxidant-1 Cu chaperon (ATOX 1) is responsible for Cu transport in the cytosol and its delivery to two major energy-dependent copper pumps: ATP7A and ATP7B (Davies et al., 2016). These are expressed in most tissues and are involved in Cu transport through a variety of interdependent mechanisms and regulatory events, including catalytic ATPase activity, Cu-induced trafficking, posttranslational modifications, and protein-protein interactions (Gaetke et al., 2014). ATPase 1 has a ubiquitous distribution and is present in multiple cell types, except for hepatocytes, whereas ATPB is present uniformly and is responsible of excretion of Cu into bile from hepatocytes. When Cu is present in excess, ATP7A translocate to the plasma membrane and effluxes Cu into the extracellular environment, to prevent its intracellular balance, leading to Cu deficiency (Menkes disease, due to mutation in ATP7A) or overload (Wilson disease, due to mutation in ATP7B) (Braiterman et al., 2014). Studies have shown that mammals have evolved the ability to sense and retain Cu in organs, however, the mechanisms are not fully understood. The high concentrations of cellular Cu ions can also increase accumulation and toxicity. Mutations in ATP7A or ATP7B, or in the proteins that regulate their transport, impair their exit from the Golgi or subsequent return to it. This may impair the metallothionein genes (MT) to scavenge the excess and toxic copper ions (Chen et al., 2020). Metallothionein's are proteins that play a crucial role in metal homeostasis, including Cu, by binding and sequestering excess Cu, hence preventing it from participating in Fenton-like reactions that generate reactive oxygen species and

cause oxidative damage (An et al., 2022). When copper transport is disrupted, the increased Cu levels may overwhelm the capacity of Mt's to effectively scavenge the excess copper, leading to increased copper toxicity (Atrián-Blasco et al., 2017). Further details on MT's are presented in the proceeding section (see section 1.4). The general mechanism of cellular Cu transport in eukaryotic cells with special reference to teleost fish is shown in Figure 2.

Protein	Acronym	Putative copper function
Membrane		
Copper transport 1	CTR1	Plasma membrane uptake
Copper transport 1	CTR2	Endosomal pump
Divalent metal transporter 1	DMT1	Cu^{2+} or Cu^{1+} import
ATPase	ATPase	Cu^{2+} import
Amyloid precursor protein	APP	Cu homeostasis
Intracellular		
Menkes disease protein	ATP7A	Enzyme biosynthesis and efflux
Wilson disease protein	ATP7B	Enzyme biosynthesis and efflux
Metallothionein	MT	Storage and chaperone
Copper metabolism MURR domain	COMMD1	Hepatic efflux
X – linked inhibitor of apoptosis	XIAP	Hepatic efflux
Chaperones		
Antioxidant	ATOX1	ATP7A, ATP7B target
Copper chaperone SOD	CCS	SOD target
Assembly factor 17 for CCO	COX17	CCO target
Assembly factor 11 for CCO	COX11	COX2 Site B
Suppressor of COX17 mutation 1	SCO1	COX1 Site A
Suppressor of COX17 mutation 2	SCO2	COX1 Site A

ATPase – adenosine triphosphatase; SOD copper – zinc superoxide dismutase; CCO – cytochrome c oxidase

Table 1. Copper binding proteins involved in Cu transport. Expression pattern of these Cu transporters and chaperones may be useful in determining Cu status in cells (Prohaska, 2008).

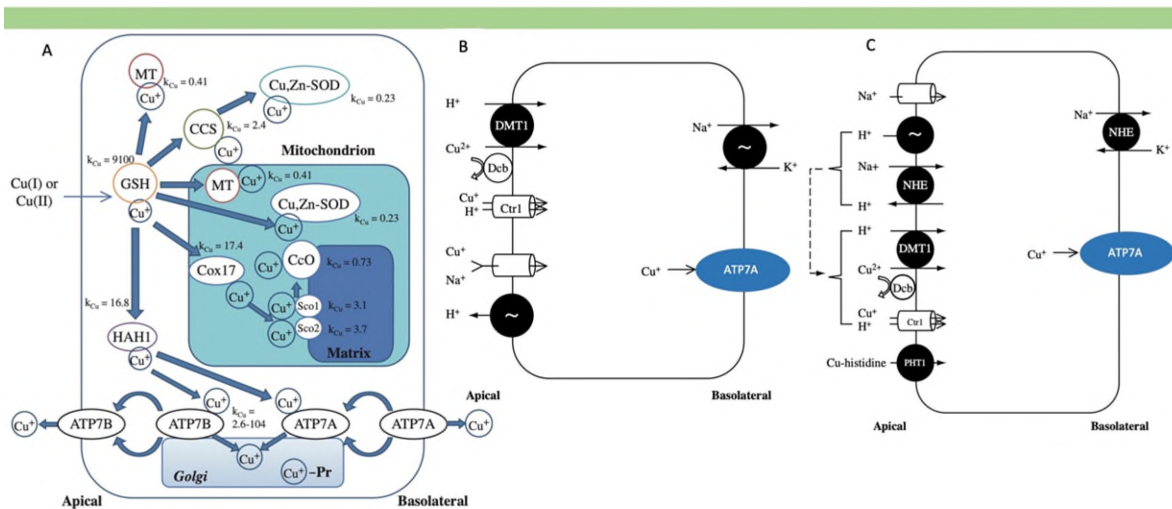

Figure 2. Schematic representation of (A) Cu transport pathways in eukaryotic cells;, (B) suggested Cu uptake pathways across (freshwater) fish gills; (C) suggested Cu uptake pathways across the intestinal epithelium of teleost fish (source: Grosell, 2011).

1.1.5 Copper Toxicity

Measurements of total copper concentrations in the environment (e.g., surface water, sediments, etc.) cannot be used to predict risks to organisms. This is due to the varying bioavailability of Cu, where sometimes only small portion of the total amount of Cu are bioavailable, while at other times, larger portions are available (De Jonge et al., 2016). Bioavailability of Cu is critical for its uptake by aquatic organims, and is influenced by several key factors, including chemical form, speciation, pH, water hardness, temperature complexation with organic matter, sediments and microbial exudates (Johnson, 1989; Mebane, 2023). Most organisms have regulation mechanisms to protect themselves from metal-induced toxicity. These mechanisms regulate metal status through metal-binding proteins at the transcriptional, translational, and enzymatic levels (Dameron & Harrison, 1998; Gaetke et al., 2014). The presence of a complex system of metal ion transporters and chaperones to regulate Cu homeostasis ensures that essential proteins are supplied with Cu without causing cellular damage (Gaetke et al., 2014). Disruption of Cu homeostasis has been associated with tissue damage and a range of diseases (Bleackley and Macgillivray, 2011; de Romana et al., 2011). In addition to direct interaction with essential macromolecules and minerals, several mechanisms, in particular free radical-induced

oxidative damage through the formation of reactive oxygen species (ROS), can cause damage to DNA and proteins and eventually cell death (Stern et al., 2007). One of the most important consequences of ROS formation is lipid peroxidation, via peroxy radicals which may damage cells by altering membrane fluidity and permeability or by directly attacking DNA and other intracellular molecules such as proteins (Gaetke et al., 2014; Lesiów et al., 2019). Cu affects other specific mechanisms and pathways such as neuronal activity, and other vital cellular processes (Giampietro et al., 2018; Gromadzka et al., 2020). In humans, Menkes disease and Wilson disease are the best-known diseases caused by a disturbance in Cu metabolism, resulting from mutations in ATP7A and ATP7B, respectively. The study of these diseases has greatly improved the understanding of the molecular mechanisms of Cu metabolism.

1.2. Cadmium

1.2.1 Sources of cadmium in environment

Cadmium (Cd) is a relative rare, naturally occurring element of great environmental concern. Cd is similar to Zn in terms of physicochemical and geological properties and in the geosphere these are found associated with each other as well. In addition, Cd is also found in lead and copper ores (ATSDR 2012). Cd occurs in the environment from both natural and anthropogenic sources. Naturally a large amount of Cd is released from volcanic eruptions, soil erosion, weathering of rocks, and from the emissions of forest fires. The rest of the Cd enters the environment through human activities such as mining, smelting, agriculture, combustion of fossil fuels, incineration, industrial and municipal discharge (US EPA, 2016). Anthropogenic sources of Cd account for more than 90% of total Cd in surface waters (US EPA, 2016). In the general human population, Cd exposure occurs through multiple pathways, including direct ingestion or dermal contact with contaminated soil, drinking, eating, and breathing (Cao et al., 2015; Yousaf et al., 2016). Although dietary intake has been recognized as the most important pathway of Cd exposure (Helmfrid et al., 2015; Chanpiwat et al., 2019), drinking has been shown to play the most important role in some mining areas (Lee et al., 2005). WHO has set a Cd limit of 0.003 mg/L in drinking water and 5ng/m^3 in air. However, drinking water, may contain Cd levels above the acceptable drinking water standard of 0.005 mg/L, (ATSDR,2012) especially when soft or acidic water tends to dissolve Cd from plumbing and levels are elevated in water that stagnates in household plumbing.

These sources have not been reported to cause clinical cadmium poisoning, but even low levels of contamination can contribute to the accumulation of cadmium in the body.

1.2.2 Environmental levels and exposures to cadmium

Cd is emitted to the atmosphere mainly in elemental form and as oxide. From some sources it is also emitted as a sulphide (coal combustion and non-ferrous metal production) or chloride (waste incineration). Cd (as oxide, sulfate, and chloride) occurs in the air as particles or vapours and is deposited (wet or dry) on soils and water surfaces (ATSDR, 2012). In the soil, Cd and its compounds can migrate depending on several factors such as pH and the amount of organic matter. In general, Cd binds strongly to organic matter where it is trapped in the soil and may be taken up by plants and eventually enters the food web. In aquatic systems, Cd exists as hydrated ion or as ionic complexes with other inorganic or organic substances. Insoluble forms of Cd are deposited in sediments while soluble forms migrate in water and may be taken up and accumulated by aquatic organisms. Compared to freshwater higher levels of calcium and chloride in seawater compete and complex with Cd to a greater extent, possibly making it less bioavailable to aquatic life. Cd concentrations in surface and ground water are generally less than 1 µg/L or 1 part per billion (ppb) (ATSDR, 2012). Dissolved Cd concentrations in polluted surface waters often range between 2-3 µg/L (0.01 – 0.02) (USEPA, 2016). In Europe, the level of dissolved Cd levels in stream water ranges from <0.002 to 67 µg/L (Knapen et al. 2004; Bervoets et al. 2005; Pan et al., 2010). Regardless of the route of exposure, it is steadily accumulated, retained in tissues and remains accumulated throughout life.

1.2.3 Cadmium toxicity

Cd is a non-essential metal with no known biological functions. Cd is one of the most toxic metals with multidirectional toxicity (Genchi et al., 2020). Due to its extreme toxicity, it is categorised as a group 1 human carcinogen by the International Agency for Research on Cancer of the World Health Organisation (IARC, 1993), group - 2a carcinogen by Environmental protection agency (EPA) and 1B carcinogen by the European Chemical Agency (ECA) (ATSDR 2012). Cd has been reported to exert nephrotoxic, genotoxic and immunotoxic effects (Lippmann, 2000; Risso-de-faverney et al., 2001; Satarug et al., 2018). In addition, it causes strong teratogenic and adverse effects on human male and female reproduction and affects pregnancy or its outcome (Zhu et al., 2020; Liu et al., 2021). Cd

enters mitochondria through calcium channels and after binding to peptide and protein thiol groups it interferes with oxidative phosphorylation, cellular ATP levels and alters membrane permeability.

The toxic effects of Cd are understood to be largely due to its free ionic form which primary disrupts the homeostasis of Ca^{2+}, as well as Na^{+} and K^{+}, and finally causes the leakage of Fe^{2+} which leads to indirect oxidative damage due to increase in the production of reactive oxygen species (ROS) and several other effects (Sarkar, et al., 2013; USEPA, 2016) resulting in blockage of DNA repair mechanism, induction of cellular proliferation, inhibition of apoptotic mechanism (Zarros et al., 2008; Rani et al., 2014; Genchi et al., 2020). As a defence response, Cd toxicity is closely associated with the expression of metallothionein's (MTs), a small family of cysteine-rich metal-binding proteins involved in Cd detoxification, anti-apoptotic and anti-oxidative functions (Sabolić et al., 2013) (Figure 3). However, intracellular detoxification of Cd may not be restricted to MTs alone. Glutathione an abundant intracellular thiol has important antioxidant properties against Cd stress (Mendoza-Cozatl et al., 2005).

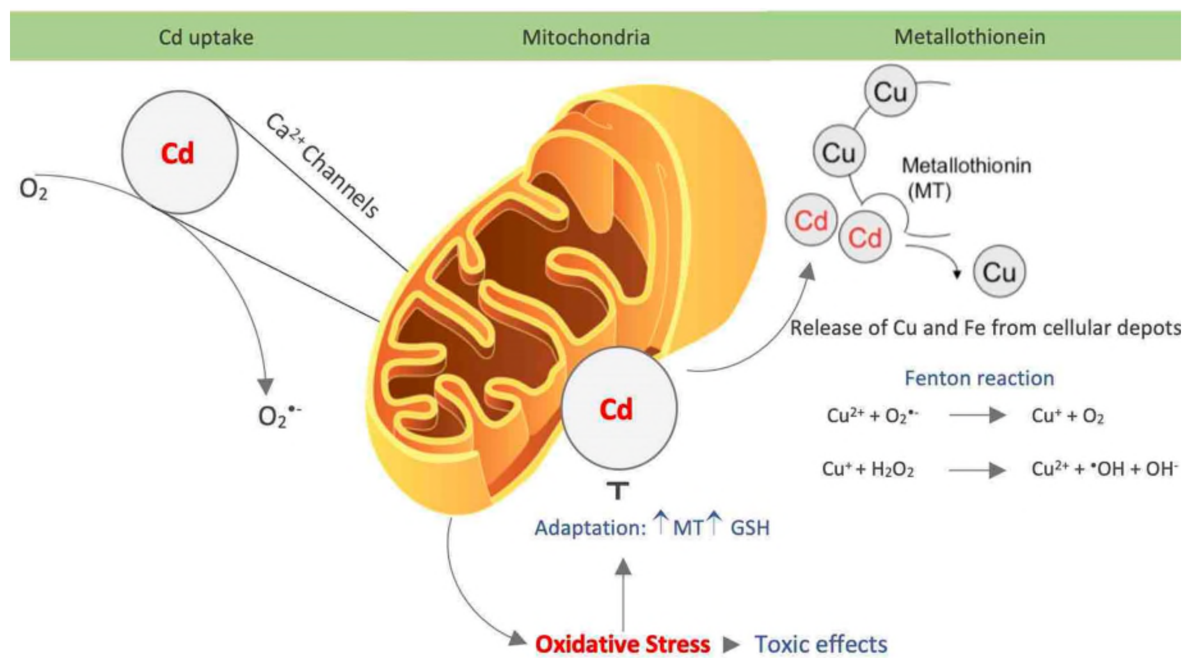

Figure 3. Cd and oxidative stress. Cd is a non – redox metal. Several mechanisms for generation of ROS have been proposed. Exposure to Cd induces expression of MT and triggers adaptation mechanism towards oxidative damage (Frank et al., 2010).

1.3 Oxidative stress

Copper and cadmium are known to cause oxidative stress (Patra et al., 2011; Zhang et al., 2012; Brown et al., 2017; Faraji et al., 2019; Chwalba et al., 2023). Oxidative stress is a physiological state that arises from an imbalance between the production of reactive oxygen species (ROS) and organisms antioxidant capacity to neutralize them or repair the resultant damage (Mailloux & Harper, 2012; Pizzino et al., 2017; Sies, 2019). ROS are highly reactive molecules containing oxygen, such as superoxide anion ($O2^{-}$), hydroxyl radical (OH•), and hydrogen peroxide (H_2O_2), which are produced naturally as byproducts of the cellular metabolism, particularly in processes involving oxygen, such as aerobic respiration (Juan et al., 2021; Tiwari et a., 2002). While ROS play essential roles in cellular signaling, immune response, and defense against pathogens under normal conditions (de Almeida et al., 2022; Sinenko et al., 2021), their excessive accumulation beyond the antioxidant capacity of organism, they can cause oxidative damage to lipids, proteins, and DNA, leading to disruptions in cellular functions and structures (Sinenko et al., 2021). Among the various signaling pathways known to be affected by metal toxicity, oxidative stress stands out as a critical pathway that initiates a cascade of cellular dysfunction in response to metal exposure (Chen et al., 2018; Valko et al., 2005; Jomová & Valko, 2011). The impact of oxidative stress has been shown to extend beyond individual cellular processes, influencing various downstream effects of metal toxicity (Chen et al., 2018). For instance, oxidative stress can lead to the formation of DNA lesions, including strand breaks and chromosomal aberrations, which affects the genomic integrity and stability (Cho et al., 2022; Sedelnikova et al., 2010). Concurrently, oxidative stress-induced protein oxidation can result in structural alterations and loss of function, disrupting cellular processes dependent on properly functioning proteins (Boguszewska-Mańkowska, 2015; Cecarini et al., 2007). In addition, oxidative stress has been shown to activate inflammatory pathways, leading to the production of pro-inflammatory mediators and recruitment of immune cells, exacerbating tissue inflammation and damage (Lugrin et al., 2013; Zuo et al., 2019). Furthermore, oxidative stress-mediated modulation of cellular signaling pathways influences cell fate decisions, impacting overall tissue homeostasis (Iqbal et al., 2024; Riegger et al., 2023).

In this thesis, an investigation was undertaken to understand the molecular responses triggered by metal exposure and the mechanisms involved, filling in some of the knowledge gaps we currently encounter. Recognizing the central role of oxidative stress in mediating

the cellular response to metal-induced damage, we chose oxidative stress as a focal point of our study. Alterations in the oxidative stress markers, such as, ROS levels, DNA damage, reduced glutathione levels and activity of antioxidant enzymes can serve as early indicators of metal-induced toxicity, providing valuable insights into the initial stages of cellular damage (He et al., 2017). In parallel, genes associated with apoptosis, cell cycle and DNA damage, and reproduction were also studied. The selection of genes was made based on their physiological, or functional significance in the context of metal toxicity as detailed in Table 2. The rationale behind their inclusion lies in their link with oxidative stress and their role in orchestrating cellular fate in response to metal toxicity (Iqbal et al., 2024; Riegger et al., 2023). In addition, we extended our investigation into developmental toxicity, focusing on genes relevant to early life-stage development in the three animal models. In addition, several ion transport genes, including those related to copper (such as ctr1), calcium (serca), zinc (zip), and cadmium (mt) were analyzed. Since ctr1 is primarily responsible for the uptake of copper (70% of uptake in human cells) into the cell from the extracellular environment (Gupta et al., 2009; Ohrvik et al., 2014; Wen et al., 2021), we also attempted to analyze genes involved in the intracellular trafficking and distribution of copper ions (including ATP7A and ATP7B), Divalent metal transporters (DMTs) as well as, the genes involved in the transport of sodium, magnesium, chloride and potassium ions due to their important role in cellular homeostasis (Hoorn et al., 2020; Morth, 2011). However, our efforts were hindered by technical difficulties, leading to unsuccessful outcomes in analyzing these genes in the three tested species.

Table 2. Target genes studied for investigating metal – induced toxicity in three animal models.

Gene symbol	Gene name	Gene function	References
aif1	Apoptosis-inducing factor 1	A mitochondrial protein that plays a role in apoptosis.	Liu et al., 2016; Sevrioukova, 2011
api5	Apoptosis Inhibitor 5	Blocks apoptotic pathway under various physiological and pathological conditions to promote cell survival by inhibiting activation of caspases.	Chen et al., 2021; Cho et al., 2014
bax	Bcl-2 Associated X-protein	Pro-apoptotic protein, promotes mitochondrial-mediated apoptosis.	Pawlowski et al., 2000; Qian et al., 2022
bcl2	B-cell lymphoma 2, Apoptosis regulator	Anti-apoptotic protein, regulates mitochondrial outer membrane permeabilization.	Cook et al., 1999; Qian et al., 2022
casp3	Caspase 3	Plays a central role in the execution phase of apoptosis; activated downstream of both the intrinsic (mitochondrial) and extrinsic (death receptor) pathways of apoptosis.	Brentnall et al., 2013; McIlwain et al., 2013
casp9	Caspase 9	Initiator caspase that functions at the convergence point of the intrinsic apoptotic pathway, also known as the mitochondrial pathway.	Brentnall et al., 2013; McIlwain et al., 2013
cat	Catalase	Neutralizes ROS and maintains cellular redox balance.	Nandi et al., 2019; Kang et al., 2012
cdc23	Cell division cycle 23	A critical regulator of cell cycle and cell growth.	Zhang et al., 2011; Xie et al., 2024
cp	Ceruloplasmin	Involved in iron metabolism by oxidizing ferrous iron ($Fe2+$) to ferric iron ($Fe3+$), which facilitates its binding to transferrin for transport in the blood. Cp also functions as an antioxidant.	Chen et al., 2017; Hellman et al., 2002
ctr1	Copper Transporter 1	Copper transporter, involved in copper uptake into cells.	Kuo et al., 2001; Ohrvik & Thiele 2015
cyclin-b	Cyclin b	Regulatory protein that controls the progression of cells through the cell cycle; Binds to cyclin-dependent kinase 1 (CDK1) forming maturation-promoting factor (MPF), which is necessary for the transition from G2 phase to the M-phase .	Ashwell et al ., 2012; Malumbres, 2020

Gene symbol	Gene name	Gene function	References
ecac	Epithelial Calcium Channel	Calcium ion channel, involved in calcium transport across epithelial cells.	Qui & Hogstrand et al., 2004; Vennekens et al., 2001
gadd45	Growth Arrest and DNA Damage Inducible Protein 45	Involved in DNA repair, cell cycle regulation, and apoptosis.	Huang et al., 2024; Tamura et al., 2012
gpx	Glutathione Peroxidase	Catalyzes the reduction of hydrogen peroxide and lipid peroxides.	Lubos et al., 2011; Pei et al., 2023
gsr	Glutathione-Disulfide	Detoxification of electrophilic compounds and ROS by catalyzing recycling of glutathione.	Hayes et al, 2005; Yan et al., 2013
gstm[1]	Glutathione S-Transferase Mu	Detoxification of electrophilic compounds and ROS by catalyzing the conjugation of glutathione.	Guo et al., 2020; Zhang et al., 2022
hhex	Hematopoietically Expressed Homeobox	Transcription factor, involved in hematopoiesis and development.	Jackson et al., 2023; Paz et al., 2010
hsp70	Heat Shock Protein 70	Chaperone protein involved in protein folding, refolding, and degradation processes.	Mayer & Bukau, 2005; Pratt et al., 2010
hsp90	Heat Shock Protein 90	Chaperone protein involved in protein folding and stabilization.	Pearl & Prodromou, 2006; Pratt et al., 2010
mt2	Metallothionein-2	Metal-binding protein, involved in metal detoxification and homeostasis; Primarily induced in response to Cu exposure, as well as Cd and Zn or oxidative stress.	Chen et al., 2014; Takashahi et al., 2015;
mt-a	Metallothionein-a	Biomarker of metal exposure; Studied in the context of metal toxicity in *Daphnia magna*, in response to exposure to Cu, Cd, and Zn; Specifically induced by cadmium even at low concentrations, and other metals at higher concentrations.	Assselman et al., 2013; Poynton et al., 2008
neuro D	Neurogenic Differentiation Factor	Transcription factor, involved in neuronal differentiation.	Chae et al., 2004; Tutukova et al., 2021
ngn1	Neurogenin 1	Transcription factor, involved in neurogenesis and neuronal differentiation.	Christensen et al., 2020; Ma et al., 1998
notch2	Notch homolog 2	Involved in cell-cell communication and embryonic development; Essential for the development of various tissues and organs, including nervous system, cardiovascular system, and immune system.	Suzuki et al., 2018; Zhou et al. 2022

[1] The mu class of enzymes function in the detoxification of electrophilic compounds, including carcinogens, therapeutic drugs, environmental toxins and products of oxidative stress, by conjugation with glutathione (accessible via: GSTM1 glutathione S-transferase mu 1 [Homo sapiens (human)] - Gene - NCBI (nih.gov)).

Gene symbol	Gene name	Gene function	References
opsin	Opsin	Involved in phototransduction, the process by which light is converted into electrical signals that are transmitted to the brain for visual perception.	Shichida, & Matsuyama, 2009; Terakita, 2005
p53	Tumor Protein p53	Tumor suppressor protein, involved in DNA repair, cell cycle regulation, and apoptosis.	Liu and Xu, 2011; Liu et al., 2020
pax8	Paired Box 8	Transcription factor, involved in organogenesis, particularly thyroid development.	Mansouri et al., 1998; Di Palma et al., 2013
rad17like	RAD17-like protein	Member of the RAD17 family that play a role in the DNA damage response by activating the DNA damage checkpoint and facilitating DNA repair.	Post et al., 2003; Siede et al., 1996
rad51	Recombinase activity gene 51	Key protein in homologous recombination repair of DNA double-strand breaks.	Bonilla et al., 2020; Wang et al., 2022
serca	Sarco/endoplasmic reticulum calcium ATPase	Pumps calcium ions from the cytoplasm into the lumen of the sarcoplasmic reticulum (in muscle cells) or endoplasmic reticulum (in other cell types), thereby maintaining low cytoplasmic calcium concentrations and facilitating muscle relaxation, cell signaling, and calcium storage.	Caspersen et al., 2000; Xu & Van Remmen, 2021
shha	Sonic Hedgehog Protein	Signaling protein, involved in embryonic development and tissue patterning.	Kotaro et al., 2017; Sasai et al., 2019
sod1/ CuZn-sod	Superoxide Dismutase 1 (Copper/Zinc Superoxide Dismutase)	Primarily located within the cytoplasm of cells; Converts superoxide radicals to hydrogen peroxide and oxygen.	Bunton-Stasyshyn et al., 2015; Wang et al., 2018; Xu et al., 2022
sod2/ Mn-sod	Superoxide Dismutase 2 (Manganese Superoxide Dismutase)	Located in mitochondria, converts superoxide radicals to hydrogen peroxide and oxygen.	Flynn & Melov, 2013; Wang et al., 2018
sod3	Superoxide Dismutase 3 (Extracellular Superoxide Dismutase)	Extracellular superoxide dismutase, involved in scavenging superoxide radicals.	Wang et al., 2018; Wert et al., 2018
vri	Vrille	Involved in regulating the timing of circadian rhythms and coordinating behavioral and physiological responses to daily light-dark cycles.	Gunawardhana et al., 2021; Kato et al., 2022
vtg	Vitellogenin	Serves as a major source of amino acids and lipids for developing oocytes during vitellogenesis, the process of yolk formation in eggs.	Biscotti et al., 2018; Li & Zhang, 2017

Gene symbol	Gene name	Gene function	References
xpa	Xeroderma pigmentosum group A	Regulates cell cycle; Plays a role in nucleotide excision repair (NER) at damage sites as a scaffold for other proteins in order to ensure that the damages are appropriately excised.	Fadda et al., 2015; Krasikova et al., 2022
zip1	Zinc Transporter 1	Zinc transporter, involved in zinc uptake into cells.	Dufner-Beattie et al., 2006; Hara et al., 2017
zip9	Zinc transporter 9	Involved in zinc homeostasis by transporting zinc ions across the plasma membrane and regulating their intracellular concentrations.	Converse & Thomas, 2020; Taniguchi et al., 2013

1.4 Defense mechanisms

As mentioned above, metals such as Cu and Cd have the ability to induce oxidative stress in living organisms, triggering a cascade of harmful effects that can compromise cellular integrity and overall health. When organisms are exposed to elevated levels of these metals, there is a risk of oxidative damage to essential biomolecules such as proteins, lipids and DNA, leading to impaired physiological function and increased susceptibility to disease (Singh et al., 2019; Haider et al., 2023). In response, organisms have developed a variety of defence mechanisms to mitigate the toxic effects of metals and maintain internal homeostasis. One of these mechanisms is the production of antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione, which work synergistically to neutralise reactive oxygen species (ROS) generated during metal exposure (Jomova et al., 2023). In addition, organisms utilise metal detoxification pathways employing metallothionines to bind, metabolise or remove excess metals from their system (Davis & Cousins, 2000, Ruttkay-Nedecky, et al., 2013).

Metallothioneins (MTs) are a class of low- molecular weight proteins, typically ranging from 500 to 14,000 daltons and act as a first line of defense against metal toxicity (Bakiu et al., 2022). These proteins are characterised by their high cysteine content (almost 30% of their amino acid composition) and are universally present across diverse organisms, from

prokaryotes to eukaryotes (Sigel, 2009; Donald et al., 2015; Wang et al., 2016; Parameswari et al., 2021; Purać et al., 2021). MTs are categorised into four main groups; MT-I, MT-II, MT-III and MT-IV, and each containing several isoforms (Thirumoorthy et al., 2007; Ruttkay-Nedecky, et al., 2013; Donald et al., 2015; Wang et al., 2016). While MTs share a common structural feature of having a high cysteine content, different MT isoforms can exhibit distinct characteristics and functions. The differences between various MT isoforms can be attributed to several factors, including the number and location of metal-responsive elements (MREs) in their promoter regions, as well as the specific amino acid sequence and resulting protein structure (Asselman et al., 2013). For instance, a study on *Daphnia pulex* found that the four MT homologs in this organism displayed time- and homolog-dependent transcription patterns in response to Cu and Cd exposure (Assselman et al., 2013). Furthermore, the metal-binding properties of different MT isoforms can vary, with some exhibiting a higher affinity for certain metals over others (Asselman et al., 2013. This is exemplified by the isolation and characterization of two distinct metallothionein isoforms, MT-I and MT-II, from the kidneys of the striped dolphin, *Stenella coeruleoalba,* which were found to have similar but not identical amino acid compositions and metal-binding capacities (Kwohn et al., 1986).

In mammalian, MTs may contain 61–68 amino acids, out of which 20 are cysteines (Kägi et al., 1988; Romero-Isart & Vasak, 2002; Ruttkay-Nedecky, et al., 2013). In humans, MT-1 and -2 (also called 2a) have high sequence homology, are often described together as MT-1/2 and are found and actively expressed in nearly all tissues (Donald et al., 2015; Jamrozik et al., 2023). MT-3 is known to exhibit slightly different characteristics compared to MT-1 and MT-2 and is expressed mostly in brain tissue, and also in heart, kidneys and reproductive organs (Ruttkay-Nedecky, et al., 2013; Donald et al., 2015). MT-4 is uniquely found in stratified squamous epithelial tissues and is prominently expressed in keratinocytes (Donald et al., 2015). These genes are located on chromosome 16 in a cluster and comprise 16 identified genes, five of which are pseudogenes, as these do not have ability to encode functional proteins (Moleirinho et al., 2011). The MT-I protein include many subtypes, which are encoded by a series of 13 MT-1 genes. The known active MT-1 genes are MT-1A, -1B, -1E, -1F, -1G, -1H, -1M and -1X. The remaining MT-I genes (MT-1C, -1D, -1I, -1J and 1L) are pseudogenes and are not expressed in humans (Ruttkay-Nedecky, et al., 2013).

Metallothionines are known to be involved in diverse cellular functions (Davis & Cousins, 2000, Ruttkay-Nedecky, et al., 2013). The role of MTs in the maintaining homeostasis of essential metal ion is mostly investigated, due to their high affinity for these metals (Ruttkay-Nedecky, et al., 2013). MTs maintain the cellular pool of essential metal ions such as, Zn and Cu and to mitigate the toxic effects of xenobiotic metals including Cd, mercury (Hg), arsenic (Ar) and lead (Pb) (Freisinger & Vašák, 2012; Wong et al., 2017). In particular, MTs play a central role in the metabolism and kinetics of metals such as Cd and Cu by facilitating their transport within organisms (Donald et al., 2015; Calvo et al., 2017), thereby influence critical cellular processes (Krężel & Maret, 2017; Purać et al., 2021). The binding affinity of MTs is due to the presence of thiol groups (-SH) in their cysteine residues forming bond with the metal ions (Sigel, 2009; Wang et al., 2016; Purać et al., 2021). These are composed of two domains: α and β. The α domain (which contains 11 cysteine residues) binds four Zn^{2+}, four Cd^{2+} or six Cu^{+} ions, while the β domain (which contains nine cysteine residues) binds three Cd^{2+}, three Zn^{2+} or six Cu^{+} ions.

In addition, MTs possess antioxidant function and can directly scavenge ROS, including superoxide radicals and hydroxyl radicals (Kumari et al., 1998; Wang et al., 2016; Ettinger, 2017; Purać et al., 2021). The thiol groups (-SH) present in MTs can react with ROS, neutralizing their reactivity and preventing oxidative damage to cellular components (Kumari et al., 1998; Wang et al., 2016; Ettinger, 2017; Purać et al., 2021). Furthermore, Mt is a part of intracellular antioxidant defense system, functioning as a secondary antioxidant to coordinate the activity of primary antioxidants including superoxide dismutase (SOD), catalases (CAT) and glutathione in maintaining the cellular redox state (Brulle et al., 2006; Jamrozik et al., 2023). Superoxide dismutase acts as the first line of defense by converting superoxide radicals into hydrogen peroxide and oxygen. Subsequently, catalase, catalyses the breakdown of hydrogen peroxide into water and oxygen, thereby preventing the formation of highly reactive hydroxyl radicals (see Figure 4). Glutathione, a key antioxidant molecule, works in coordination with enzymes like glutathione peroxidase to neutralize reactive oxygen species and maintain cellular redox balance (Sharma et al., 2012; Kumar et al., 2022). It participates in redox reactions as a reducing agent and maintains the cellular redox balance by cycling between its reduced (GSH) and oxidized (glutathione disulfide, GSSG) forms (Jozefczak et al., 2012; Aquilano et al., 2014).

A crucial aspect of cellular defense against oxidative stress is DNA repair. DNA repair mechanisms play a pivotal role in maintaining genomic stability and integrity in the face of oxidative insults. Various DNA repair genes are activated in response to oxidative stress to rectify DNA damage caused by ROS (Jia et al., 2016; Zhong et al., 2017) (see Table 2). In addition to DNA repair mechanisms, heat shock proteins (HSPs) are another essential component of the cellular defense system against oxidative stress (see Table 2). HSPs, particularly HSP70 and HSP90, are induced in response to various stressors, including oxidative stress, and play a critical role in protecting cells from oxidative damage (Nakaya et al., 2007; Cole & Meyers, 2010; Ikwegbue et al., 2018; Sulzbacher et al., 2020).

In our study, we focused on investigating the effects of Cu and Cd exposure on MT levels in three tested species: zebrafish, daphnia and planarians. This choice was made due to the well-established role of Mt in metal homeostasis, detoxification and combating oxidative stress. Through this comparative approach, our aim was to determine, how varying exposure scenarios of Cu and Cd influence both the expression and functionality of MT, thus elucidate its adaptive significance in different biological contexts.

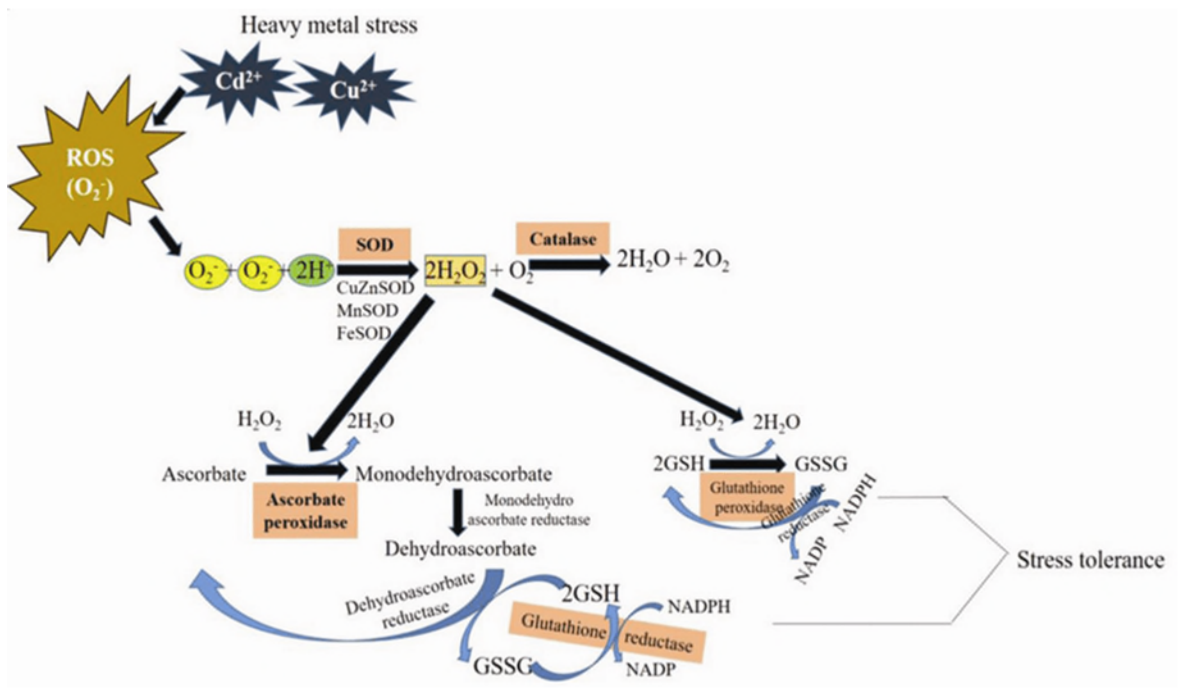

Figure 4. Mechanism of important antioxidant enzymes-superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase-helping in metal stress tolerance (source: Somasundaram et al., 2018)

1.5 Metal mixture toxicity

It is well-known that, in a polluted environment, metals often occur as complex mixtures, resulting in simultaneous exposure of organisms to multiple metals (Bloch et al., 2023). Both observational and experimental evidence show that such co-exposure can lead to combined effects that are different from those expected when considering the individual chemicals (Pilehvar, et al., 2019; Majid et al., 2022). Within such mixtures, metals can undergo chemical interactions that influence their uptake and mode of action, depending on their chemical properties (such as their oxidation states and binding affinities) and mechanisms of action (including their ability to disrupt cellular processes or induce oxidative stress) (Meyer et al., 2015; Kaushal et al., 2018). As a result, predicting the toxicity of metal mixtures is more complicated than it is for the individual metals. They can result in additive, synergistic, or antagonistic effects, impacting their overall toxicity and environmental fate (Vijver et al., 2011; Van Genderen et al., 2015). Metals that interact synergistically produce larger effects (more-than-additive) than the expected effect when present alone, while others may behave antagonistically and produce lower effects (less-than-additive) than would be expected from the combined exposure (Vijver et al., 2011; von Stackelberg et al., 2015). In additive interactions, the combined effects are equal to the sum of their individual effects, indicating no interaction between the metals in the mixture (Iin et al., 2016; Roell et al., 2017; Li et al., 2020). The additive model is used to predict the combined toxicity of metal mixtures based on the assumption that the individual toxicities of each metal can be summed to estimate the overall toxicity of the mixture (Gao et al., 2016; Nys et al., 2016). While additive effects occur when metals do not interact synergistically or antagonistically, they are less common compared to the latter two types of interactions (Vijver et al., 2011; Martin et al., 2021). However, the prevalence of these effects appears to correlate significantly with the specific combination of metals tested and the chosen toxicological endpoint (Vijver et al., 2011). Regulatory authorities are particularly concerned about synergistic interactions due to their potential to exacerbate toxicity beyond individual chemical effects (Cedergreen, 2014). Thus, a comprehensive understanding of the mechanisms and implications of synergistic effects is vital for developing effective regulatory measures.

Given the complexity of the combined effects of metal mixtures, researchers have increasingly turned to advanced modelling techniques to better understand and predict

their behaviour (SCHER, 2012). For ecological assessments of metals, the USEPA recommends using either the concentration addition (CA) model or the independent action (IA) models (USEPA 2007). In the CA model, substances are assumed to have a similar mode of action, and each component within the mixture is assumed to contribute independently to the overall toxicity, i.e., without interactions between the components (Loewe and Muischnek, 1926; Altenburger et al., 2000; Escher et al., 2020). The model is based on the principle that the combined effect of chemicals at a given concentration is simply the sum of their individual effects and is therefore also known as the dose addition model (Hadrup et al., 2013). Whilst CA offers a straightforward approach and is often used as a basis for comparison, it may underestimate or overestimate toxicity if synergistic or antagonistic interactions are present (SCHER, 2012). The IA model, also known as response addition model, takes into account the potential interactions between the components of the mixture (Bliss, 1939; Backhaus et al., 2000; Escher et al., 2020). It assumes that the effects of the individual chemicals within the mixture are independent but can have a multiplicative effect. In other words, the IA model predicts the combined effect based on the assumption that each chemical acts independently, with their effects multiplying rather than adding up (Bart et al., 2021). This model is particularly useful for evaluating synergistic or antagonistic interactions within the mixture.

The choice between the using CA or IA models depend on the scenario and the nature of the chemicals involved. In the situations, where chemicals in a mixture have similar modes of action or biological sites, the concentration addition model may be more appropriate (Parrella et al., 2014). On the other hand, if the chemicals have independent mechanisms of action or different biological sites, the independent action model may be more suitable (Nys et al., 2016). However, it is often hard to conceptually select the model that better fits the data, given that chemicals may have multiple and/or unspecified modes of action in the studied organisms. From previous studies on chemical mixtures, different deviation patterns from CA and IA have been reported (Lock and Janssen, 2002) as well as more complex deviations such as dose level and dose ratio dependent responses (Jonker et al., 2004). In terms of predicting toxicity, the differences between the predictions between CA and IA does not exceed five, depending on the specific components and their relative potencies (Hadrup et al., 2015).

The CA model is more commonly recommended and used in ecotoxicology compared to the IA model (Lock and Janssen, 2002). The CA model is preferred because it is considered a more conservative approach that can be used as a default when the mode of action (MoA) of the mixture components is unknown or dissimilar. As the CA model assumes additivity of components in a mixture, even if they have different MoAs, it is suggested as a reasonable model in the worst-case scenario of mixture toxicity (Lock and Janssen, 2002; Martin et al., 2021). However, it's essential to note that determining the presence and magnitude of synergistic interactions can be challenging, requiring detailed knowledge of the mechanisms of action and interactions between chemicals in the mixture. Therefore, while IA may be more suitable for worst-case scenarios involving synergism, it also comes with greater complexity and data requirements compared to CA. In scenarios where synergistic interactions between mixture components are of concern, the IA model may provide a more accurate prediction of combined toxicity. This is because the IA model accounts for potential interactions between mixture components, including synergistic effects, and therefore may offer a more precautionary approach in risk assessment (Cedergreen, 2014). Nonetheless, the CA model remains widely used due to its simplicity, ease of implementation, and extensive validation in both laboratory studies and environmental monitoring, making it a well-established and accepted approach in ecotoxicology and regulatory frameworks. However, there is still a pressing need for more research into the combined action of mixtures (Spurgeon et al., 2003; He and Van Gestel, 2015; Drakvik et al., 2020).

1.6 Current regulatory regimes on the risk assessment of mixtures

The toxicity of metals is an area of concern to both the public and regulatory agencies around the world. Regulatory agencies worldwide have established water quality standards, guidelines, and regulations, such as USEPA's Clean Water Act (Public Law 95-217) and the European Union's Water Framework Directive (DIRECTIVE 2013/39/EU) to manage contamination of water. These standards typically include concentration limits based on scientific assessments of toxicity, exposure pathways, and ecological impacts. For example, the USEPA has established both acute and chronic aquatic ambient water quality ,which are the highest concentration of specific pollutants or parameters in water that are not expected to pose a significant risk to the majority of species in a given environment (USSEPA, 2024). The acute criterion for Cu, designed to protect aquatic life from short-term exposure, is set at 2.33 μg/L and the chronic criterion, intended to protect aquatic life from long-term

exposure, is set at 1.45 µg/L (normalised to a water hardness of 85 mg/L) (Liao et al., 2023). The value for Cd is set at 1.8 µg/L for both acute and chronic exposures (normalised to a water hardness of 100 mg/L) (USEPA, 2024). The WFD (2000/60/EC) sets environmental quality standards (EQS) for priority substances, including Cu and Cd, in surface waters. These standards are based on the concentrations that should not be exceeded to prevent adverse effects on aquatic ecosystems. The Flemish (Belgium) EQS for Cu in freshwater of is 7 µg/L, while for Cd, a priority substance the EQS ranges between 0.08 – 1.5 µg/L depending upon water hardness[2] (available via: TITEL II VAN HET VLAREN BIJLAGEN).

Despite environmental regulations on metal emissions, metal pollution remains an environmental challenge. In the past decades, understanding of the potential effects of metal toxicities on aquatic ecosystems has significantly advanced (Hama et al., 2023; Singh et al., 2023). However, it also needs to be realized that organisms in the real environment are simultaneously exposed to mixtures of metals and other compounds. Metals are generally regulated on a single metal basis (USEPA, 2013). Mixture toxicity is mentioned in various guidelines but is not directly included in the regulations for metals (Meyer et al., 2015). The regulatory approach based on individual metals was because of the complexity in assessing and modelling the toxicity of metal mixtures. In the past years, there has been relatively intensive research into the toxicity of mixtures (OECD 2018; Bopp et al., 2018, 2019; Drakvik et al. 2020; Escher et al., 2022; Bloch et al., 2023).

Given the importance of the mixture effects, there are urgent calls to include mixtures in future risk assessments (Kortenkamp et al., 2009: Drakvik et al., 2020) but there is no consensus yet on how to include the toxicity of metal mixtures in the risk assessment process with the concentration addition model being the most realistic option for the time being. Although there is ample evidence of exposure and adverse effects of mixtures, there is a significant need to develop methods and find advanced solutions for how we establish causality between exposure and effects. Moreover, given an almost infinite number of real

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2 For Cadmium and its compounds the EQS values vary depending on the hardness of the water as specified in five class categories (Class 1: < 40 mg $CaCO_3$/l, Class 2: 40 to < 50 mg $CaCO_3$/l, Class 3: 50 to < 100 mg $CaCO_3$/l, Class 4: 100 to < 200 mg $CaCO_3$/l and Class 5: ≥ 200 mg $CaCO_3$/l) (Eu Commission, 2013, available via: Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policyText with EEA relevance (europa.eu)

mixtures, it is not possible to study all of them. Simplifications need to be made to aid prediction of exposure and effects estimates.

Our current regulatory systems are not designed to deal with simultaneous exposure to a wide range of chemicals (Drakvik et al., 2020). In Europe, efforts are being made to effectively regulate chemical mixtures, but no legislation in European Union explicitly regulates the toxicity of metal mixtures (Meyer et al., 2015) due to a lack of a harmonised, consistent approach to risk assessment and risk management of mixtures across regulatory areas. In 2007 the European Commission commissioned a study to review the state of scientific knowledge and regulatory approaches on mixtures. The study was completed in 2009 and a report entitled State of the Art Report on Mixture Toxicity (Kortenkamp et al, 2009) was produced. The European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) has released reports focusing on the aquatic toxicity of chemical mixtures, indicating a growing interest in understanding the effects of mixtures on the environment (Backhaus & Faust, 2012). The EU's 7th Environment Action Programme of 2013 set the target of ensuring that by 2020 the combination effect of chemicals is effectively addressed in all relevant Union legislation as part of a new strategy for a non-toxic environment (EC, 2013). In 2016, the EU's Joint Research Centre (JRC) published a review of case studies assessing the risks of chemical mixtures to humans and the environment (Bopp et al, 2016). The assessments were based on Concentration Addition (CA). The review found evidence of combined exposure to chemicals regulated under different legislation and evidence that such chemicals may cause similar effects or have a similar mode of action. The review concluded that the risk assessment of mixtures under different regulatory regimes should be further investigated.

Several organisations, including the Organisation for Economic Cooperation and Development (OECD), the European Food Safety Authority (EFSA), and the World Health Organisation (WHO), have published guidance and considerations for risk assessment of mixtures, providing an updated description of approaches, methods, specific challenges, and research needs (WHO, 2017; OECD, 2018; EFSA, 2019;). Specific challenges for risk assessment and management of chemical mixtures have recently been summarised in a publication by the EU Commission's Joint Research Centre (JRC) (Bopp et al., 2019). This report also suggests pathways based on novel methodologies.

EFSA published its "Guidance on harmonised methodologies for the assessment of risks to human health, animal health and the environment from combined exposure to multiple chemicals" in March 2019 (EFSA, 2019). The EFSA guidance describes harmonised risk assessment methodologies for the risk assessment of mixtures for all relevant areas within EFSA's remit, i.e., human health, animal health and environmental health. Similar to the OECD guidelines, EFSA proposes a framework for risk assessment of mixtures that includes problem formulation and tiered approaches for hazard identification and characterisation, exposure assessment and risk characterisation. Both whole-mixture and component-based approaches are considered. The overall objective of the Horizon 2020 funded EuroMix project (available via: https://cordis.europa.eu/project/id/633172) was to develop methods and tools for mixture risk assessment.

In 2021, the European Union (EU) published Regulation (EU) 2021/849 as Adaptation to Technical and Scientific Progress (ATP) amending Part 3 of Annex VI to Regulation (EC) 1272/2008 on classification, labelling, and packaging of substances and mixtures (CLP Regulation, consolidated version until May 2021). The latest ATP entered into force on 17 June 2021 and applies from 17 December 2022. Substances and mixtures may be classified, labelled, and packaged in accordance with this amendment from the date of entry into force. The European Union's Green Deal and zero-pollution ambition aim to address the existing gaps in chemical mixture risk assessment by providing scientific grounds to support the implementation of regulatory measures within the EU (Escher et al., 2022). Collaborative projects and initiatives, such as the European Partnership for Chemicals Risk Assessment (PARC), are working towards advancing research on chemical mixtures and developing innovative approaches to predict adverse health effects resulting from exposure to mixtures (Marx-Stoelting et al., 2023). Despite advancements in the field, challenges persist in conducting in-depth assessments of specific chemical mixtures due to knowledge and data gaps. These gaps can hinder the comprehensive evaluation of mixture effects, emphasizing the need for further research and methodologies to address these gaps effectively.

2. Species sensitivity

Species sensitivity refers to the varying responses exhibited by different species when exposed to toxic substances or environmental stressors and may be over several orders of magnitude (Kooijman, 1987). This variation in species sensitivity has been related to

behavioral, physiological morphological and life-history traits of organisms (Rizzi et al., 2021) as well as various environmental parameters, including temperature, pH, and water hardness which can modulate the toxicity of chemicals (Collas et al., 2018). Variation in these parameters leads to differences in toxicokinetic (TK) or toxicodynamic (TD) profiles of certain toxicants (Anna-Maija et al., 2014). Toxicokinetics describes the Adsorption-Distribution-Metabolism-Excretion (ADME), which determines chemical concentrations in the organism, ultimately reaching the target site (Nyman et al., 2012). Toxicodynamics involves the processes of interaction between the chemical and the target and the resulting cascade of events that lead to phenotypic changes. Understanding species TK and TD traits has significant potential to help support the read-across of species (Rivetti et al., 2020). Consequently, understanding the different biochemical pathways that contribute to the TK and TD traits may be informative for understanding the nature and severity of species responses to chemical exposures. The assessment of species sensitivity is important in toxicological studies because different organisms can have different responses to the same chemical or pollutant (Cairns, 1980). In addition, understanding the range of sensitivities across species is necessary to accurately assess the potential impacts of contaminants and establish appropriate water quality standards (Cairns, 1980). For addressing sensitivity differences, two main approaches have been developed. The first is to use the "safety factor" (also known as uncertainty factor or assessment factor), which is a number by which an observed or estimated no observed adverse effect concentration (NOAEC) or the lowest observed effect concentration (LOAEL) or dose is divided to produce a criterion or standard that is considered safe (or has an acceptable level of risk) (Dankovic et al., 2015). This approach has become widely used in the regulation of chemicals (ECHA, 2008). In the current European Chemicals Regulation safety factors between 10 and 1,000 are applied (ECHA, 2008). These safety factors are based on how much effect data is available for a given chemical, and higher safety factors are used when less data is available.

The second, more probabilistic approach, is to use the species sensitivity distribution (SSD) approach. An SSD is a statistical distribution that describes the variation among the species in vulnerability towards toxicants (Kooijman, 1987; Posthuma et al., 2002). SSD method is used in the determination of safe concentration levels for chemical substances in both terrestrial and aquatic ecosystems and is often included in Ecological Risk Assessment (ERA) procedures (Rizzi et al., 2021) . The 5th percentile of a distribution (called the 5% hazardous concentration, HC_5) is used to derive threshold concentrations that are protective of most

species in a community (more correctly the range of sensitivities observed across a collection of taxonomically different species) (Rizzi et al., 2021). This is conventionally, the threshold for impact routinely used in SSDs, where the protection of 95% of species is ensured and the acceptance of an adverse impact is 5% (Nagai, 2016). For illustration, a species sensitive curve for arthropods exposed to dissolved Cd is shown in Figure 5. In recent years, the concept of the ecological threshold of concern (eco-TTC), an analogous to traditional human health-based TTCs has emerged as a pragmatic tool for conducting screening-level ecological risk assessments (ERAs) for ecological species (Barron et al., 2021; Rizzi et al., 2021). This method is applicable to chemicals lacking sufficient ecotoxicological data, and offers a valuable means of delineating an exposure threshold. This threshold denotes the concentration level below which the risk to aquatic organisms is considered negligible.

For generating SSDs, both acute and chronic toxicological data can be used. Chronic toxicity data are ecologically more important because organisms are usually exposed to low concentrations of contaminants over a long period of time. In principle, SSDs should therefore be based on chronic toxicity data rather than acute toxicity data. However, for many compounds there is not enough chronic data to construct SSDs and therefore acute toxicity data are sometimes used to estimate chronic values using so called acute to chronic ratios. This approach introduces considerable additional uncertainty and should be avoided. In case of metals, considerable efforts have been made to obtain chronic toxicity data for the most important metals so that in case of Cu or Cd for example robust SSDs can be constructed. The exact number of species required for SSD depend on factors such as the variability in sensitivity among species and the level of confidence desired in the assessment results. In general, a larger and more diverse dataset (≥ 8 species) can provide a more robust assessment of the potential ecological risks associated with a chemical substance (Belanger et al., 2016; Iwasaki et al., 2021). There have been technical challenges and improvements in SSD methodology over the last 20 years, driven by advances in the availability of computing technology (Fox et al., 2021). However, several studies have addressed some of the limitations, sample size issue, species representativeness and selection, test endpoints, ecological relevance, phylogenetic relationships, and exposure pathways (Dyer et al., 2006; Wang et al., 2015; Moore et al., 2019). Newman et al. (2000) examined species sensitivity data and concluded that the standard distributions such as log-logistic, log-normal, or Gompertz all have systematic deviations from the available data that are not covered by the three distributions. Moreover, the different distributions often do not fit the data very well.

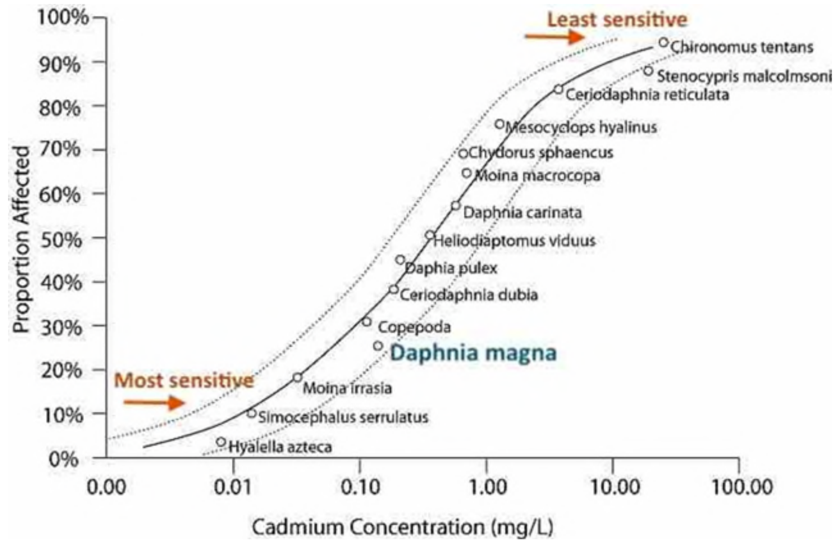

Figure 5. Species sensitive curve of acute LC_{50}s for arthropod species exposed to dissolved cadmium over 48-72 h, with 95% confidence intervals (dotted lines). Data obtained from U.S. EPA's ECOTOX database. (Source: https://www.epa.gov/caddis-vol4/ssd-plots

Early studies, such as those by Slooff and Canton (1983), highlighted the interspecific differences, emphasizing the need for a diverse array of species in toxicity testing to capture this variability. Since, SSDs are inherently descriptive, there is no real understanding of why some species are more sensitive than others. According to Slooff et al. (1983), there is no indicator species with universal sensitivity to all toxicants and that aquatic toxicity testing should include a taxonomically diverse variety of species. This concept evolved into an approach for establishing ecosystem protection goals against chemical pollution in which a protective threshold for a specified proportion of species is chosen (Van Straalen and Denneman, 1989). Fitting and using SSDs require specialized software tools for optimal accuracy and efficiency. Presently, there are at least nine such tools designed to fit SSDs using a range of methodologies, primarily emphasizing maximum likelihood estimation. Notably, the US Environmental Protection Agency (USEPA) has developed an SSD Toolbox, a comprehensive software tailored for estimating hazardous concentrations in ERAs (Fox et al., 2021). This toolbox incorporates advanced features such as model averaging and employs various statistical distributions to effectively fit data. The process involves several steps: first, compiling results from separate toxicity tests conducted on a specific chemical across diverse aquatic animal species. Second, selecting a statistical distribution believed to align with the test data and fitting it accordingly. Finally, utilizing the fitted distribution to

derive a concentration level deemed protective of a desired proportion of species within a hypothetical aquatic ecosystem.

Given the challenges associated with creating SSDs, our study does not aim to create an SSD based on these species, but rather focuses on utilizing the inherent sensitivity differences among these model organisms to explore the effects of metal mixtures across different taxa. While other species may also exhibit sensitivity to metal contamination, the selection of these three models allows for a comprehensive assessment of sensitivity across vertebrate and invertebrate taxa due to their unique characteristics, and representing different levels of biological complexity. Furthermore, through comparative analysis among these species, we aim to get insights into commonalities and unique responses in response mechanisms, therefore contributing to better understanding of species-specific vulnerabilities to metal pollution in a mixture scenario.

3. Experimental organisms

Present study was conducted on three animal models: the zebrafish *(Danio rerio),* the water flea *(Daphnia magna)* and the planarian flatworm *(Schmidtea mediterranea).* The morphology, ecology, and role of these animal models in toxicology is described below.

3.1 Zebrafish (*Danio rerio*): Morphology, ecology, and role in toxicology

Zebrafish *(Danio rerio)* is a freshwater fish (adult length 4 – 5 cm), belonging to the family Cyprinidae. It naturally inhabits the tropical and subtropical freshwater bodies of South and Southeast Asia. The common name *"zebrafish"* originates from the presence of uniformly pigmented horizontal stripes on the lateral sides of the body, which are reminiscent of a zebra's stripes. Zebrafish is omnivorous and mainly feeds on zooplankton, phytoplankton, and insects (McClure et al., 2006).

Zebrafish males can be distinguished from females by a torpedo – shaped body presence of golden stripes between the blue stripes. Females are plumper, having a wider whitish belly with silver stripes along with blue. Zebrafish have short generation, and during embryonic development, undergoes various stages such as blastula, gastrula, and segmentation, eventually forming distinct organ systems (D'Costa & Shepherd, 2009) (see Figure 6). Around

24 hours post-fertilization, the embryo hatches from its protective chorion and becomes a free-swimming larva. It typically achieve reproductive maturity within 3 to 6 months (Hoo et al, 2016)

Over the past years, zebrafish has emerged as a prominent and fast expanding model organism in biological and biomedical research (Veldman & Lin, 2008; Khan & Alhewairini, 2018; Knapen et al., 2020; Villeneuve & Knapen 2022). It is also a recommended animal model in OECD toxicity guidelines (OECD, 2019) and is widely used in toxicological research (Spitsbergen and Kent, 2003; Hill et al., 2005; Dai et al., 2014). Zebrafish has multiple characteristics that make it a suitable animal model. These include small size, high fertility, fast embryonic development, transparent embryos which enables examination of developmental stages in real time (Khan & Alhewairini, 2018). As a vertebrate, zebrafish has several physiological and genetic similarities with humans, including aspects of brain, digestive tract, vascular system, musculature and innate immune system organisation and functioning (Khan, 2018). Advances have been made in using zebrafish as a model for development of enteric nervous system, angiogenesis, regeneration, and oncology (Xiang et al., 2009; Khan, 2018). In addition, to the above-mentioned characteristics, zebrafish require little space and have low maintenance cost. Adult and embryonic zebrafish are widely used in monitoring toxic metals in aquatic systems as well as in the assessment of nanomaterials and endocrine disruptors (Dai et al., 2014; Cristiano et al., 2019). Metals can affect zebrafish development causing various teratogenic effects, behavioural changes, alter biochemical and molecular changes related to enzyme activity and/or transcriptional alterations in different biomarker genes (Sevcikova et al., 2011; de Lima et al., 2013; Sonnack, et al., 2015; Douglas et al., 2018; Monaco et al., 2017; Liao et al., 2021 Sánchez-Olivares et al., 2021).

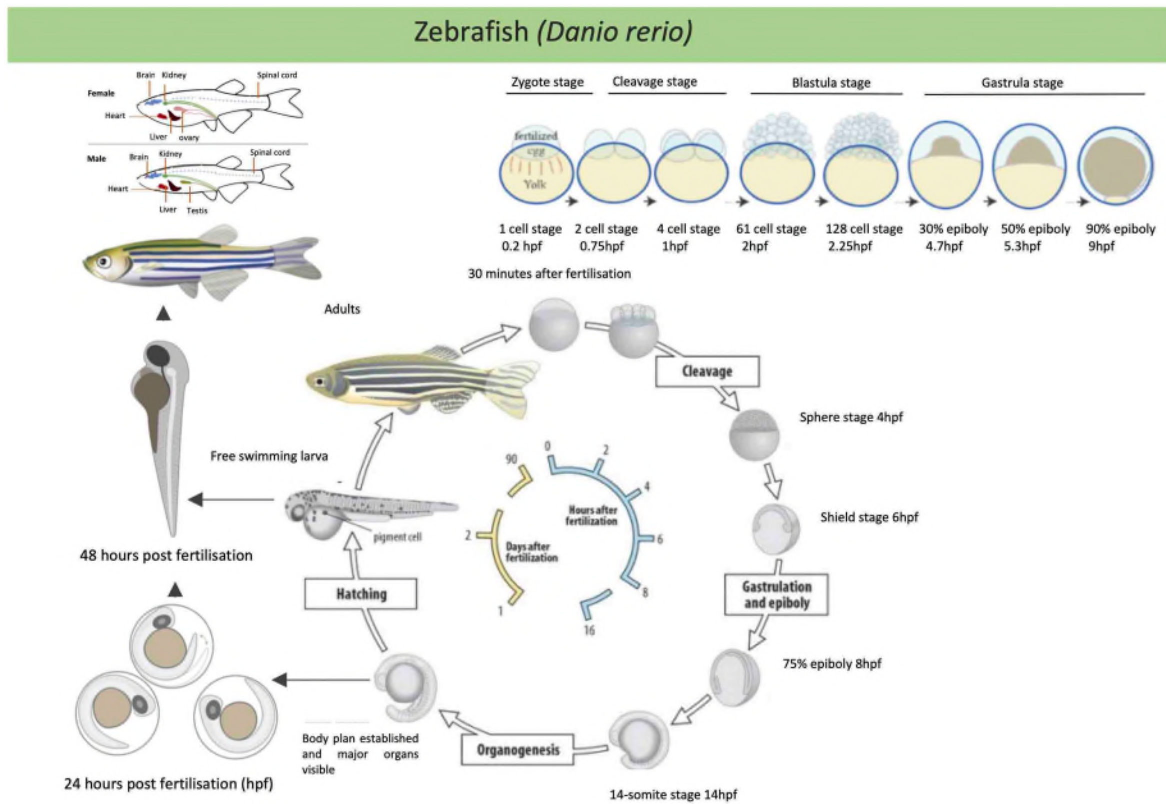

Figure 6. Diagrammatic representation of zebrafish life cycle. Zebrafish lifecycle is divided into four life stages: embryo, larva, juvenile, and adult. Zebrafish develops rapidly from one-cell zygote and completes the full life cycle in 90 days (Images adapted from: https://invivobiosystems.com/crispr/zebrafish-101-a-white-paper/ , https://salepeaket.live/product_details/44013932.html).

3.2 Water flea (*Daphnia magna*): Morphology, ecology, and role in toxicology

Daphnia magna is a small (adult length 1.5 – 5mm) planktonic crustacean, found in a wide range of lentic aquatic habitats (Ebert, 2005; Gewin, 2011; Seda and Petrusek, 2011). The common name "*water flea"* originates from its typical hopping movements arising from the beating of large swim antenna (Stollewerk, 2010). *D. magna* have a transparent body, enclosed by an uncalcified shell known as the carapace, with multiple tissue layers surrounding the thorax and abdomen. They have two pairs of antennae, the first two being sensory in function and the second are modified for swimming. The head possesses two compound eyes and a brain connected to the eye and gut opening.

Males are distinguished from females by a smaller body, larger antennules, modified post-abdomen and first legs (Ebert, 2005). *D. magna* represent the largest species in the genus

and are regarded as a keystone species in lentic freshwater ecosystems. These are filter–feeders, and primary feed on algae, and secondarily feed on the suspended particles that can be retained by their filtering apparatus (>1µm) (Gillis et al., 2005). *D. magna* are extensively used in eco-toxicological studies due to their numerous advantages such as fast generation time, low cost, easy culturing, and handling under laboratory settings (Seda, 2011). *D. magna* have adaptability to a variety of adverse environmental conditions such as drought, cold and food scarcity (Stollewerk, 2010). Depending on the prevailing conditions, *Daphnia* reproduce asexually through parthenogenesis or sexually by producing resting haploid eggs (Stollewerk, 2010) (see Figure 7). The parthenogenetically produced eggs are deposited in a brood pouch, in which they develop and mature in around 3 days (at 20 ^{0}C) and are released into the environment (Ebert, 1994). The neonates are mature after 6 to 8 days during which they undergo 4-5 moulting stages (Ebert, 1994). After maturation they produce their first brood during the first week of their life which repeats every two to three days for the rest of their life. *D. magna* are sensitive to the changes in water chemistry and are most extensively used in bioassays for chemical toxicity screening and the toxicity testing of effluents from contaminated waters. These are recommended test species in OECD toxicity testing guidelines (OECD, 2004). Daphnia are an important model system for assessing the toxicity of various chemicals, including pharmaceuticals (Tkaczyk et al., 2021), pesticides (Maggio et al., 2021) and metals (Yuan et al., 2020). Metals generally have negative effects on development, population growth rate, longevity, and reproduction. In addition, transgenic daphnia that are very sensitive to (very low level) metals have been produced by metallothionine gene editing to facilitate the monitoring of water quality (Arao et al., 2020). Metallothionein gene responds to metals and could, therefore, be useful for the classification of toxicants (e.g., to indicate whether the adverse outcome is due to metal toxicity or any other stimuli such as heat, to which HSP70 respond well). Transgenic zebrafish and nematode have also been developed; however, they only respond to higher metal concentrations (Arao et al., 2020).

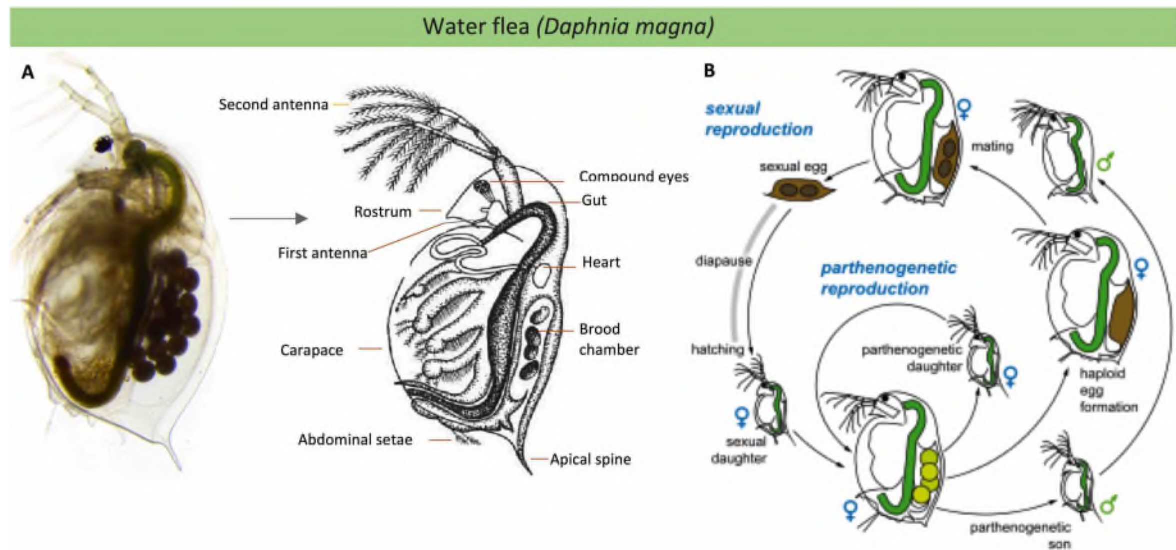

Figure 7. Diagrammatic representation of *Daphnia magna.* The figure shows (A) Morphology (B) Life cycle of *D. magna. Daphnia* have a lifecycle based on "cyclical parthenogenesis", alternating between parthenogenetic (asexual) and sexual reproduction. Daphnia undergoes a direct development which results in juveniles that are morphologically similar to their adults. (source: De Bonis et al., 2013; Elbert, 2022)

3.3 Planarian (*Schmidtea mediterranea*): Morphology, ecology, and role in toxicology

Planarians are free-living freshwater acoelomate, triploblastic, bilaterally symmetrical animals, belonging to the class Turbellaria[3]. Freshwater planarians exhibit a worldwide distribution, representing benthic communities or inhabit floating vegetation in freshwater environments (Noreña et al., 2015). The body structure of freshwater planarians is simple but has some unique biological characteristics. The body plan of planarians shows three major axis of polarity: dorsal/ventral, anterior/posterior and medial/latera (Martín-Durán et al., 2012; Durant et al., 2016). These axes provide spatial orientation to the organism and determine various aspects of its anatomy and behaviour. Hundreds of planarian species exist in marine, freshwater and terrestrial environments worldwide (Ivankovic et al., 2019). They generally vary in size from only a few millimetres in many marine trichilids to 10-30 mm in freshwater forms. Despite their simple external appearance, their anatomy is quite complex (see Figure 8). They possess protonephridia as excretory system and mesenchyme filling the

[3] Turbellaria are characterized by their soft, unsegmented bodies and are commonly found in freshwater and marine environments. Turbellarians are known for their diverse feeding habits, which can range from scavenging and predation to parasitism. They play a significant role in aquatic ecosystems as both predators and prey, contributing to the overall biodiversity and ecological balance of their habitats (Rohde, 1994) .

space between the various organs of the body (Jordan and Verma, 2007; Felix et al., 2019). Planarians have no specialized circulatory organs for the transport of oxygen or digested food to the tissues (Cebria, 2016; Lewallen & Burggren, 2020). As such, oxygen is taken up through the entire body wall, while nutrients diffuse from the gut to the surrounding tissues (Lee, 2020). The triple-branched gut is a blind sac connected to a muscular pharynx and located in a pouch or sac approximately in the middle of the body. By muscular contraction the pharynx is pushed out of the mouth, penetrating the body of the prey, and then, by peristaltic action, taking up fluids and small pieces of tissue (Abel et al., 2021; Lee, 2022). Generally, there is one pharynx, one mouth opening and no anus. The nervous system is dominated by a pair of ventral nerve cords that thicken in the head portion of the animal to form the brain or cerebral ganglion (Cebria, 2008; Lee, 2020). The head also contains the eyes and the auricles, which are the main sensory structures.

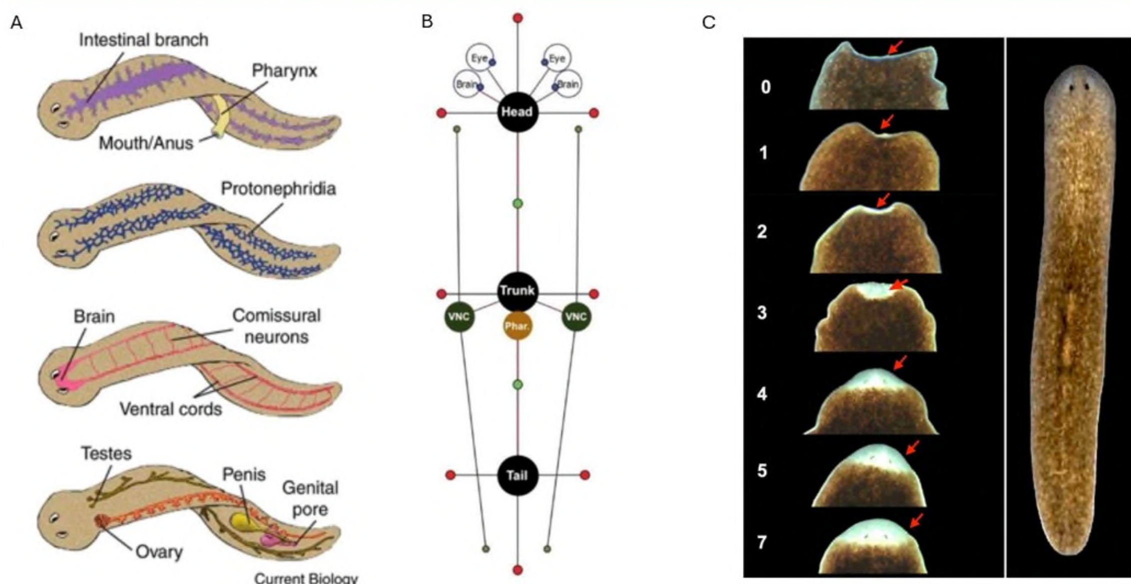

Figure 8 (A) From top to down; the figures shows digestive system, excretory system, nervous system, and reproductive system, (B) position of body parts) (C) regenerating head fragment. Regeneration involves the formation of new tissue at the wound site via proliferation of dividing neoblasts (a homogeneous stem cell population), resulting in the formation of blastema (white pigmented part; indicated by red arrow) and the remodelling of pre-existing tissues to restore symmetry and proportion (morphallaxis) (source: https://www.researchgate.net/publication/267783498_Then_in_the_Quick_guide, Newmark & Alvarado (2002) .

Planarians are hermaphroditic animals, meaning that each animal has a complete set of male and female reproductive organs. They have different modes of sexual and asexual reproduction. The sexual strains are cross – fertilizing hermaphrodites whereas the asexual

strains reproduce by fission (Hyman, 1951; Kobayashi and Hoshi, 2002; Newmark and Sánchez Alvarado, 2000. Planarians have received increasing scientific attention over the years because of their regenerating capacities (Sánchez Alvarado, 2003). Planarians are carnivores and are reported to consume living or dead arthropods (e.g., insect larvae and crustaceans), annelids (e.g., oligochaetes), and molluscs (e.g., gastropods) (Abel et al., 2021). One of the important characteristics of planarians is their great ability to survive long periods of starvation, ranging from a few months to well over a year, depending on the species (Abel et al., 2021). Prolonged starvation, however, causes remarkable degeneration of the organs, except the nervous system, which remains unaffected (Felix et al., 2019). Therefore, the severely starved worms seem to have a disproportionall large head, because the post-pharyngeal part of the body is greatly reduced. Freshwater planarians are an important component of the aquatic fauna of unpolluted streams or lake. They are often used to study the toxicity of anthropogenically generated environmental pollutants and have been suggested as invertebrate bioindicators of water quality (Knakievicz, 2014; Wu and Li, 2018). They have been known to displays distinct behavioral traits mediated by sensitive perception of environmental cues (Deochand et al., 2018).

Planarians are increasingly used in studies related to teratogenicity and tumorigenicity (Hagstrom et al., 2016), neurotoxicity (Hagstrom et al., 2015), carcinogenicity (Stevens et al., 2017) and chemical toxicity, including the toxicity of metals such as Cd, Cu, mercury (Hg) and aluminium (Al) due to their sensitivity towards metals (Kim et al., 2021). Several studies have reported various morphological, locomotor and behavioural changes in different planarians after exposure to Cu and/or Cd by analysing different biomarkers such as oxidative stress responses, regeneration performance, abnormal cell proliferation and the amount of cells showing DNA damage (Wu et al., 2014; Kim et al., 2021; Majid et al., 2022).

S*chmidtea mediterranea* is a freshwater non – parasitic flatworm (adult length approx. 20mm), which has emerged as a unique animal model for regeneration research. It is a simple metazoan with robust and rapid whole-body regeneration and ease to manipulate experimentally (Newmark and Sánchez Alvarado, 2002). In addition, it can be sufficiently multiplied in laboratory and requires low maintenance cost (Rompolas et al., 2009). The high regenerative capacity of S. *mediterranea* are largely derived from a large pool of adult pluripotent stem cells, called neoblasts, which are distributed throughout the mesenchyme except in front of the photoreceptors and pharynx (Morgan, 1998; Reddien and Sánchez

Alvarado, 2004) and account for 20-30% of all the cells. (Baguñà and Romero, 1981, Zhu and Pearson, 2016, Ivankovic, 2019). The stem cells are mitotically active and constantly enable *S. mediterranea* to grow the lost body parts throughout their lifetimes, as well as maintain the tissue integrity (Davies et al., 2017). Besides being a classical regeneration tool, *S. mediterranea* are one of the best characterised organisms used in developmental biology, stem cell biology, ageing and toxicological screenings (Newmark and Sánchez, 2002; Hagstrom et al., 2015, Stevens et al., 2017, Fincher et al., 2018).

4. Comparative toxicity test of three species

The selection of exposure concentrations for the three test species involved consideration of several factors, including species-specific sensitivities and the need to capture a range of sub-lethal effects. A comparison of acute Cu and Cd toxicities among *Danio rerio*, *Daphnia magna* and planarians based on the data included in the UESEPA Ecotox database show that the differences among species (interspecific) are large as well as within a given species (intraspecific) are also large. The latter type of variation is reasonably well understood and relates to the differences in metal bioavailability (e.g., metal speciation and water hardness effect) and genetic differences in sensitivity among populations or strains of the same species. However, the differences among species are far less understood and related to their differences in structural and functional organisation which in turn results in differences in exposure, accumulation, and internal processing. Taken together all these factors and conditions results in orders of magnitude of differences in sensitivity among species and even within a species among exposure conditions and populations. Furthermore, the number of ecotoxicity data available for the different species is very different and very well documented for *Daphnia magna*, acceptable for *Danio rerio* and very limited for Planarians. The LC_{50} results presented in Figures 9A and 9B compare the acute toxicity data extracted from the EcoTox database for Cu and Cd for the three species (for the Planarians the data of different species are presented together given the limited dataset. The overall acute LC/EC50 results can be summarized as follows, for Cu: Planarians, median=0.40 mg/l (6.3 µM) with 25 % percentile 0.22 and 75 % percentile 3.4, minimum 0.19 and maximum 6.2 mg/l (n=6), *Daphnia magna,* median=0.046 mg/l (0.72 µM) with 25 % percentile 0.023 and 75 % percentile 0.19, minimum 0.0005 and maximum 1.4 mg/l (n=489) and *Danio rerio*, median=0.14 mg/l (2.2 µm) with 25 % percentile 0.081 and 75 % percentile 0.21, minimum 0.012 and maximum 0.65 mg/l (n=24) and for Cd: Planarians, median=25 mg/l (220.2 µM)

(with 25 % percentile 4.4 and 75 % percentile 55, minimum 3.0 and maximum 80 mg/l (n=6), *Daphnia magna*, median=0.049 mg/l (0.55 µM) with 25 % percentile 0.020 and 75 % percentile 0.12, minimum 0.0016 and maximum 3.8 mg/l (n=216) and *Danio rerio*, median=3.8 mg/l (33.8 µM) with 25 % percentile 1.7 and 75 % percentile 6.5, minimum 0.12 and maximum 30 mg/l (n=15).

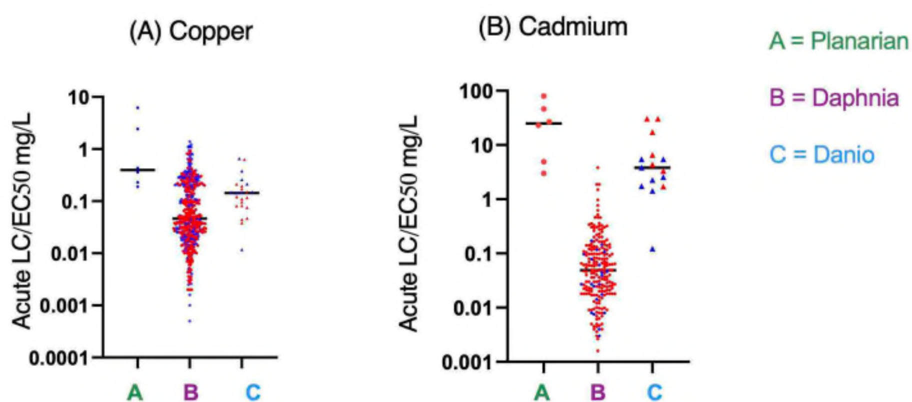

Figure 9. Comparison of the acute toxicity data (EC/LC_{50}) extracted from the EcoTox database for (A) Cu and (B) Cd for the three species (for the Planarians the data of different species are presented together given the limited dataset. Red = based on total metal concentration and Blue = dissolved metal concentration.

In the existing literature, exposure concentrations of Cu ranging from 0.001 – 10 µM have been documented to induce sub-lethal to lethal effects in zebrafish (Komjarova & Bury, 2014; Li et al., 2024; Pilehvar et al., 2019; Santos et al. 2021; Takemura et al., 2024; Tilton et al., 2011; Zhang et al., 2023a, 2023b; Zhong et al., 2022). Similarly, exposure concentrations of Cd ranging from 0.04 – 50 µM have been reported (Al Marshoudi et al., 2023; Bian et al., 2021; Di Paola et al., 2022; Hu et al., 2022; Lin et al., 2023; Zhong et al., 2023). In the first chapter of this thesis involving zebrafish, exposure, concentrations of 0.80 µM Cu and 0.25 µM Cd were selected to induce probable sublethal effects in both adults and embryos. These concentrations corresponds to 25% of the 240-hour LC_{50}, determined based on the concentration range established by Pilehvar et al. (2020) (see Figure. 10). The selection of 0.25 µM for Cd was similarly based on the concentration range established by Pilehvar et al. (2019). In the acute toxicity test conducted by Pilehvar et al., (2019), zebrafish mortality barely reached 30% after 240 hours exposure to Cd concentrations above 20 µM due to rapid precipitation of Cd before reaching LC_{50} threshold. Consequently, LC_{50} could not be

computed, which necessitated the use of a lower concentration of Cd to prevent rapid and complete mortality in mixture scenario (Pilehvar et al 2019).

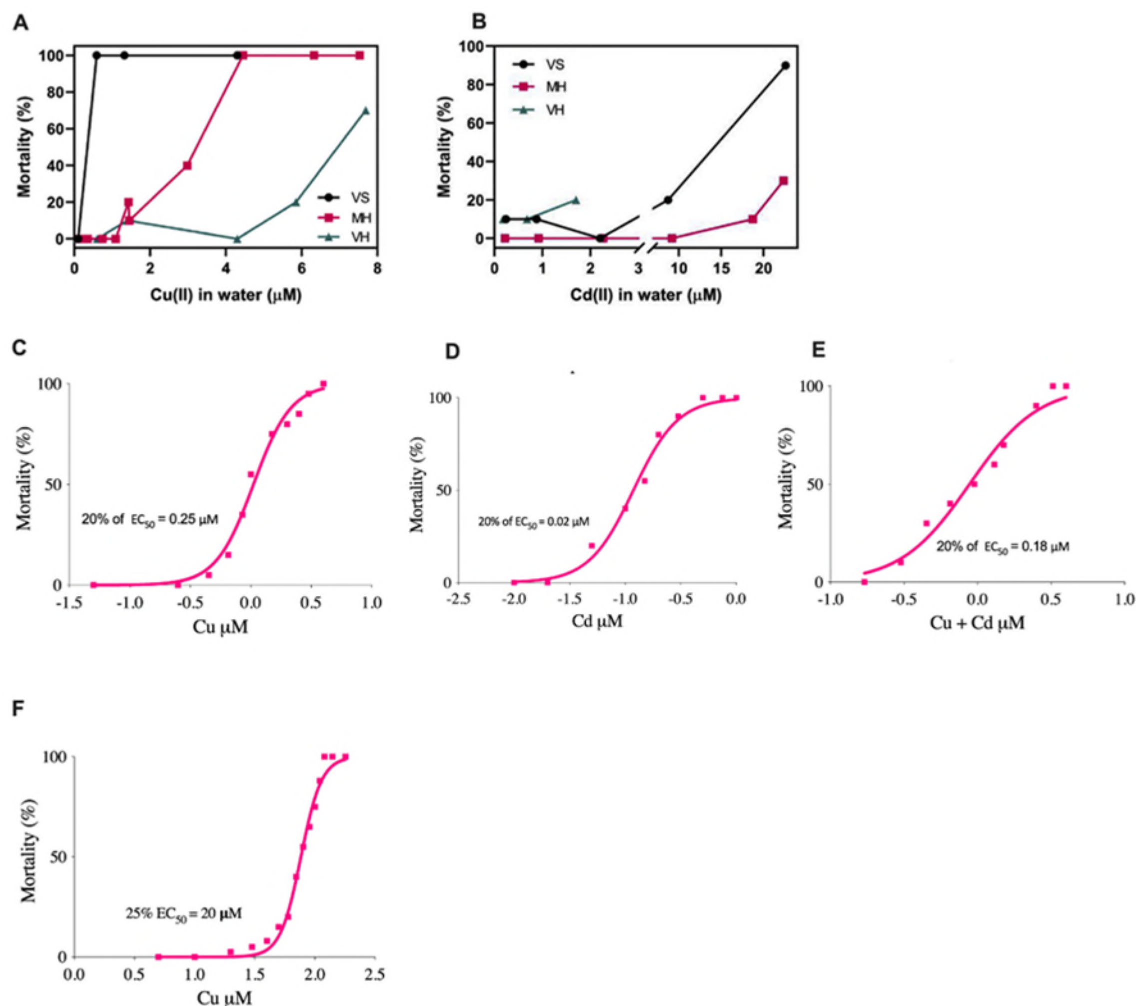

Figure 10. (A) and (B), the percentage morality of zebrafish subjected to Cu and Cd single exposures in very soft (VS), moderately hard (MH) and very hard water (VH) water for ten days (source. Pilehvar et al., 2020); (C), (D), and (E), the percentage mortality of daphnia subjected to Cu, Cd and their combination in MH water; (F) and (G), the percentage mortality of planarians subjected to Cu, and Cu + Cd combination.

The high sensitivity of daphnia is well documented in litrature and is attributed to its relatively larger surface area to body volume ratio compared to larger animals, allowing for greater absorption for waterborne toxicants including metals (Blewett et al., 2017). In addition, daphnia has a high metabolic rate, which facilitates increased uptake of chemicals across their gills (Blewett et al., 2017). Furthermore, as primary consumers these readily accumulate metals and other pollutants (Reilly et al., 2023). In the second chapter 2 of this thesis, the selected lower concentrations of 0.25 μM Cu, and 0.02 μM

Cd compared to zebrafish and planarians are considered taking into account their relatively high sensitivity to metals compared to other aquatic organisms (Reilly et al., 2023; Sadeq et al., 2019). These exposure concentrations were determined in a 48-hour static, non-renewal lethality test in accordance with the standard OECD Test Guidelines No. 202 (OECD, 2004). The test was performed, using concentration ranges of 0.15 – 3.0µM for Cu, and 0.02 – 1.0 µM for Cd. These concentrations were within the range reported in litrature(Canizares-Villanueva et al. 2000; Komjarova and Blust 2009a; Kim et al., 2017; Lari et al. 2017; Li et al., 2022; Vandenbroek et al. 2009) and were applied in a gradient to produce 0% to 100% mortality. Based on 20% of 48 h LC_{50} (95% confidence interval), a fixed concentration of 0.25 µM was selected for Cu and 0.02 µM was selected for Cd. For the combined exposure, the 20% LC_{50} corresponded to a combined dose of 0.18 µM (0.17 µM for Cu and 0.01 µM Cd) (see Figure 10).

While there is some research on the toxicity of Cu and Cd on planarians, the available studies are not as extensive compared to those on other model organisms like daphnia and zebrafish. In the third chapter of this thesis, toxicity test of Cu was performed using concentrations ranging between 0 to 180 µM at five time points (24 h, 48 h, 72 h, 1, and 2, weeks). These ranges were applied in a gradient to produce 0% to 100% mortality. Ten animals were used per condition in a blinded and randomized experimental design. The mortality was recorded at different time points, scoring zero for the dead and one for living animals. Based on the 25% of LC_{50} values of one week, a fixed concentration of 20 µM (95% confidence interval) was selected for Cu. For Cd, a concentration of 10 µM (25% of LC_{50} for one week) was chosen, based on the previous lethality test results performed by Plusquin et al., 2012. For the combined exposure, the concentration of 20 µM Cu + 10 µM Cd was selected (see Figure 10). Due to non-availability of the previously published planarian mortality data for Cd, the dose-response curve is not shown here. Metal concentrations were determined by plotting the dose-response curve using GraphPad Prism 8 statistical software GraphPad software.

In addition to species – specific sensitivities, differences in cultivation media, including variations in nutrient composition, water hardness or pH levels, can exert notable influence on the toxicological responses of organisms (De Paiva Magalhães et al., 2015; Laderrière et al., 2020). The variations in the media have the potential to alter the bioavailability and uptake of metals, consequently affecting the observed toxicity

outcomes (Brezonik et al., 2020). For instance, media components, such as calcium and magnesium can decrease the uptake of Cu and Cd in organisms by competing for absorption sites (Kies & Harms, 1989; Li et al., 2021; Wang et al., 2022). On the other hand, certain amino acids like cysteine and metallothionein peptides can form complexes with these metals, thereby facilitating their uptake into cells (Lange & Segner, 2023; Subramanian & Deepe, 2017; Viarengo et al., 1985). Furthermore, chemical constituents of culture media may vary in redox potentials, subsequently affecting the oxidation state and speciation of metal ions (Stone et al., 2021). Alterations in redox conditions possess the capacity to influence the toxicity of certain metals, particularly those that undergo redox reactions (Zhen et al., 2020). Therefore, it is important to take into account the composition of the culture medium in toxicity experiments involving metals. In our study, the composition of cultivation media slightly varied depending on the test species. We anticipate, the presence of competing ions and the alkaline pH may exert influence the toxicity of Cu and Cd in the tested species. Furthermore, the differences in water hardness, and hence the carbonate buffering system between media used for zebrafish adults, daphnia and embryos is anticipated to result in different chemical interactions, potentially altering their toxicity profiles (see Table 3).

Table 3. Composition of culture media used for D. rerio, D. magna and S. mediterranea (source, Narko et al., 2020; Pilehvar et al., 2020).

Test species	Chemical composition	Hardness ($CaCO_3$)	pH
Danio rerio (adults)	$NaHCO_3$, $CaSO_4$, $MgSO_4$, KCl, $CaCO_3$	80–100 mg/L	7.8 ± 0.2
Danio rerio (embryos)	NaCl, $CaCl_2$, $MgSO_4$, KCl, CaCO3	30 mg/L	7.5 ± 0.2
Daphnia magna (adults)	$NaHCO_3$, $CaSO_4$, $MgSO_4$, KCl, $CaCO_3$	80–100 mg/L	8.0 ± 0.2
Daphnia magna (neonates)	$NaHCO_3$, $CaSO_4$, $MgSO_4$, KCl, $CaCO_3$	80–100 mg/L	8.0 ± 0.2
Schmidtea mediterranea (adults)	NaCl, $CaCl_2$, $MgSO_4$, $MgCl_2$, KCl	Ultra-pure	7.5 ± 0.2
Schmidtea mediterranea (regenerating)	NaCl, $CaCl_2$, $MgSO_4$, $MgCl_2$, KCl	Ultra-pure	7.5 ± 0.2

General Objectives

General Objectives

The main objective of this thesis was to **investigate the individual and combined toxicity of Cu and Cd in in three animal models:** the zebrafish *(Danio rerio),* the water flea *(Daphnia magna),* and the planarian flatworm *(Schmidtea mediterranea)* and compare the outcomes at developmental and molecular levels by combining physiological and toxicological approaches. Furthermore, the aim was to **determine whether the toxicity mechanisms across the species are the same** and to provide a **better understanding of the role of exposure pathways in inducing adverse effects.**

The selection of model species is based on their common habitat as aquatic organisms, which makes them highly relevant for studying the effects of metal pollution on aquatic organisms. Each species offers unique advantages for comparing the mechanisms underlying the toxic effects (see section 3). Although these species exhibit varying degrees of biological and physiological complexity, from the simplicity of daphnia, regenerative capacity of planarians to the complexity of the zebrafish as a proxy for vertebrates. In addition, they share important characteristics that are favorable for toxicity testing, such as relatively short generation times, ease of maintenance in laboratory environments, and sensitivity to environmental stressors. Taking into account the different strengths of these animal models, our study aims to gain a comprehensive understanding of the toxicity mechanisms of Cu and Cd in single and co-exposure scenarios at the three taxonomic levels

We hypothesize that, exposure to Cu and Cd mixtures will induce oxidative stress in all three animal models, as this represents a common pathway of the metal stress. We aim to untangle how the organisms cope with induced redox differences and whether this differs between Cd, Cu, or mixed toxicity. We anticipate that the developmental processes of these organisms will be adversely affected, leading to disruption in morphogenesis and potential compromises in growth and development. We expect variations in sensitivity to the mixture among the three animal models, with daphnia exhibiting the highest susceptibility to stress. The mixture is expected to follow concentration addition (CA), where the combined toxicity is determined by the sum of their individual effects regardless of their mechanisms of action.

Chapter 1 of this thesis focuses on the embryonic and adult life stages of zebrafish *(Danio rerio),* exploring the effects of single and combined exposures of Cu and Cd. A thorough

assessment of developmental, morphological, and functional endpoints was undertaken in embryos at 96 hpf (hours post fertilization), while morphological and functional endpoints were scrutinized in adult animals seven days post-exposure. Metal accumulation and molecular responses concerning oxidative stress, apoptosis, transport, and DNA repair were investigated at both life stages, shedding light on both overarching and tailored stress reactions.

Chapter 2 compares the effects of single and combined exposures of Cu and Cd in *Daphnia magna* across different exposure pathways. The aim of separating the exposure pathways, is to evaluate the relative importance of each exposure pathway for toxicity. Adult *Daphnia magna* (20 – 21 days old) and neonates (less than 24 hours old) were exposed to Cu and Cd individually and as a binary mixture via three exposure pathways: aqueous (dissolved in water), dietary (spiked with food) and their combination (food + water), over a 7-day period. Toxicity endpoints related to survival, growth and reproduction were assessed. To explore the molecular mechanism underlying toxicity was investigated.

In Chapter 3, the freshwater planarian *Schmidtea mediterranea* was used to investigate developmental and physiological responses associated with a combined exposure to Cu and Cd. In addition, the cellular and molecular mechanisms underlying the provoked adverse effects were studied in different exposure scenarios.

At the end of this thesis, the most important findings and future perspectives are summarized, providing a comprehensive overview of the significant contribution of the study and paving the way for further research in this field.

Chapter 1

Zebrafish *(Danio rerio)*

Chapter 1. Insights into the combined toxicity of copper and cadmium in zebrafish (*Danio rerio*) embryos and adults

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In preparation for publication

Abstract

Metal pollution poses a persistent environmental challenge, impacting both ecosystems and human health. While efforts have been made to understand the mechanisms underlying the toxicological outcomes of metal pollution, there remains insufficient understanding of the key molecular and physiological events, particularly in mixed exposure scenarios and across different life stages. In this study, zebrafish (*Danio rerio*) embryos and adults were used to investigate the responses to both single and combined toxicity of copper (0.80 µM Cu) and cadmium (0.25 µM Cd). Morphological, and functional endpoints were evaluated in embryos at 96 hpf (hours post fertilisation), and in adults at 7 days exposure. Metal accumulation and molecular responses related to oxidative stress, apoptosis, metal transport, and DNA repair were studied at both life stages to gain new insights into general and specific stress responses. The results show a strongly increased sensitivity of both embryos and adult animals to the mixture compared to single metal exposures. Despite similar toxicity mechanisms, the effects were more pronounced in embryos, indicating higher sensitivity of early life stages. In addition, our data show that metal accumulation as such is not always a strong indicator of toxicity.

1. Introduction

Metal pollution has received considerable attention in past decades, due to their widespread use, subsequent discharge into the environment and the toxicities associated with them (Cuypers et al., 2010; Balali-Mood et al., 2021). Extensive research has focused on understanding the toxicity of metal mixtures, aiming to unravel the interactions and combined effects on organisms. Investigated into both acute and chronic toxicity of binary and ternary metal mixtures, including combinations of cadmium (Cd), copper (Cu), zinc (Zn), nickel (Ni), and lead (Pb), and others, have been conducted (Franklin, et. al., 2002; Meyer et al., 2015; Nys et al., 2015, 2016; Gao et al., 2016; Zeb, et al., 2017, Moyson, et. al., 2018; Zeng et al., 2019; Castaldo et al., 2020; Arreguin-Rebolledo et al., 2024). These studies have highlighted the complexity of mixture toxicity, with findings indicating that the combined effects of metals can be additive or non-additive (synergistic, or antagonistic). Furthermore, efforts to develop models that can accurately predict the toxicity of metal mixtures, accounting for factors such as bioavailability, accumulation, and ion characteristics, have been going on to improve understanding and assessment of metal mixture toxicity (Gao et al., 2016; Traudt et al., 2017; Zeng et al., 2019; Zhang et al., 2023). Despite the insights gained from these studies, certain gaps remain in current research. One significant gap lies in the limited understanding of the molecular mechanisms underlying the toxicity of metal mixtures, hindering predictions based on the molecular responses (Meyer et al. 2015). In addition, age-specific responses to metal mixtures have not been extensively studied, despite their critical importance in ecological and health risk assessment. Understanding underlying molecular mechanisms and age-specific sensitivities is crucial for accurately assessing the potential risks and better understand the developmental and physiological changes that influence the toxicological responses to metal mixtures.

To address this gap, we investigated the impact of Cu and Cd on both adults and embryos of zebrafish *(Danio rerio,)* under single and binary exposure set-ups of Cu and Cd. Zebrafish were chosen as the model organism due to their well-established status in toxicological and developmental biology research McCollum et al., 2011; Ball, et al., 2014; Roper and Tanguay, 2018; Zhao et al., 2024. The selection of Cu and Cd as toxicants is based on their frequent occurrence , their persistence in the environment, as well as their different cellular and potential modes of action, and their well-documented adverse effects their (e.g., essential versus non-essential) and their strong interactive effects (ATSDR, 2004, 2012; Kamunde and

MacPhail, 2011; Sadeq and Beckerman, 2020). Copper is a nutritionally essential metal in several physiological processes (De Romana et al., 2011; Taylor et al., 2020). It is a co-factor of many vital enzyme systems such as superoxide dismutase (SOD), ceruloplasmin, mitochondrial cytochrome *c* oxidase and tyrosinase (Hefnawy and El-Khaiat, 2015). Disturbance in Cu homeostasis results in Cu deficiency or excess, both of which lead to adverse health effects (Stern et al., 2010; Taylor et al., 2020). Copper deficiency, although rare, has been associated with neurological manifestations such as myelopathy (Jaiser et al., 2010; Lizaola-Mayo et al., 2021), and haematological abnormalities such as anaemia (Wazir et al., 2017; Jensen et al., 2019; Naz et al., 2023). Elevated Cu levels in the body can result in liver toxicity, and neurological disorders (Taylor et al., 2020). Several mechanisms have been proposed to describe Cu-induced toxicity. These mechanisms are most often associated with the ability of Cu to undergo redox cycling from oxidised (Cu^{2+}) to reduced (Cu^{+}) forms, leading to the formation of reactive oxygen species (ROS) via the Haber-Weiss reaction (Stern et al., 2007; Driessnack et al., 2017). Increased ROS levels can lead to protein oxidation, DNA damage, enzyme inhibition, lipid peroxidation, activation of apoptotic pathways and ultimately cell death (Mattie and Freedman, 2001 Gaetke et al., 2014; Lesiów et al., 2019; Villalpando-Rodriguez and Gibson, 2021).

Cadmium is a non-essential metal that poses a health risk to both plants and animals (Genchi et al., 2020). Cadmium is classified as human carcinogen (Group 1) by the International Agency for Research on Cancer (IARC, 1993), a Group - 2a carcinogen by the Environmental Protection Agency (EPA) and Group 1B carcinogen by the European Chemical Agency (ECHA) (ATSDR, 2012). Cadmium has been reported to have potential nephrotoxic, genotoxic and immunotoxic effects (Lippmann, 2000; Satarug 2018; Balali-Mood et al., 2021). In addition, it is reported to potentially cause strong teratogenic and adverse effects on human male and female reproduction and may affect pregnancy or its outcome (Massányi et al., 2020; Zhu et al., 2020; Liu et al., 2021). The toxic effects of Cd are understood to be mainly due to its free ionic form which primary disrupts the homeostasis of Ca^{2+}, as well as Na^{+} and K^{+} and eventually cause the leakage of Fe^{2+} leading to indirect oxidative damage due to increased production of reactive oxygen species (ROS) (Sarkar, et al., 2013a). Several other effects resulting in blockage of DNA repair mechanism, induction of cellular proliferation, inhibition of apoptotic mechanism have been reported (Zarros et al., 2008; Rani et al., 2014; Genchi et al., 2020).

In the current study, we aim to investigate if a combined Cu and Cd exposure induces different stress responses. Specifically, we anticipate that organisms in development react differently to metal toxicity, due to rapid cell division and differentiation processes, and different cellular defense mechanisms that are activated (Vasconcelos et al., 2010; García-Esquinas et al., 2013; Mohammad et al., 2013; LeFauve et al., 2017), while adults may exhibit distinct molecular signatures reflective of their physiological maturity and potential adaptive responses (Mohammad et al., 2013). This hypothesis is based on the previous research demonstrating synergistic toxicity of Cu and Cd on zebrafish and other fish species (Zhu et al., 2011; Ubani-Rex et al., 2017; Gao et al., 2018; (Pilehvar et al., 2019, 2020; Wu et al., 2019) as well as a well-established notion of greater sensitivity of early life stages to environmental stressors (Mohammad et al., 2013; Majid et al., 2022). We determined the key molecular events triggered in the early and adult life stages to gain insights into the toxicity mechanisms involved at each stage. Metal accumulation and transcriptional response of genes encoding proteins involved in oxidative stress, apoptosis regulation, DNA repair and transport were investigated in both embryos and adults. In embryos, developmental, morphological, and functional endpoints were also assessed. In adults, the toxic effects on different organs were compared. The results presented in this paper show stressor-specific responses to single and binary metal exposures in early and adult life stages, as well as organ-specific responses in adults.

2. Material and Method

2.1 Ethical statement

All the experimental protocols used in the study were approved by the Ethical Committee for Animal Experiments (ECD, approval 2012-11) of the University of Antwerp. Adult zebrafish maintenance and experiments were carried out in accordance with EU Directive 2010/63/EU on the protection of animals used for scientific purposes. Zebrafish embryos are not included in this Directive until the age of 120 hpf. The embryo exposure experiments did, therefore, not require approval by an Ethical Committee for Animal Testing.

2.2 Test organisms and experimental design

2.2.1 Zebrafish embryos

Unexposed adults of zebrafish (AB strain) were used for the egg production. Fish were maintained in a ZebTEC zebrafish housing system (Tecniplast, Buguggiate, Italy) containing reconstituted freshwater, prepared with Instant Ocean® Sea Salt (Blacksburg, VA, USA). The

water used for housing and breeding was maintained under the following conditions based on OECD guidelines (OECD TG 236); temperature (28 ± 1^{0}C), conductivity (500 ± 25 µS/cm, Hardness 30 mg/L $CaCO_3$, pH (7.5 ± 0.2), dissolved oxygen (O_2 ≥ 80 % saturation, 6.2 ± 0.2 mg/L O_2). About 35% of the circulating water was renewed daily to keep the levels of ammonia, nitrite and nitrate below the permissible limits (ammonium < 0.25mg/L, nitrite < 0.3mg/L and nitrate < 12.5mg/L) and were monitored twice a week using Tetratest kits (Tera Werke, Melle, Germany). An automated light cycle of 14h light:10h dark was applied. The breeding stock was fed four times per day; twice with granulated food (Biogran medium, Prodac International, Cittadella, Italy) at a rate of 1% of their mean wet weight and twice with thawed and rinsed *Artemia sp, Daphnia sp*, *Chironomidae* larvae and *Chaoborus* larvae alternately. For breeding, males and females were used in a of 2:1 ratio (OECD TG 236) and separated overnight in breeding tanks with a perforated bottom and a divider. The spawning and fertilization of eggs period was 45 minutes in the morning after the light was turned on. Immediately thereafter, eggs were collected using a disposable plastic hose and transferred into clean reconstituted freshwater.

Exposures were started 0 – 2 hpf as illustrated in Figure 1. The effects on the molecular and physiological responses in zebrafish were evaluated at a fixed sublethal concentrations of copper (0.80 µM $CuSO_4$) and cadmium (0.25 µM $CdCl_2$) and their combination (i.e. concentration addition exposure). Fresh stock solutions of one molar copper ($CuSO_4.5H_2O$) and cadmium ($CdCl_2.H_2O$) were prepared in reconstituted freshwater and subsequently diluted to attain working solutions of 0.80 µM Cu and 0.25 µM Cd. These exposure concentrations were selected to induce potential sublethal effects in both adults and embryos, and were based on the concentration range established in a prior research by Pilehvar et al. (2020) (see section 2.3). To ensure consistency in test conditions, the pH of the working solutions was adjusted to 7.5 ± 0.2 and incubated overnight at 28 ^{0}C. All the embryos (n = 120 per experiment) from the breeding couples were pooled and then randomly arranged into 5 groups. These groups were initially unexposed and designated for subsequent exposures. Among these groups, the first group was designated to be used as a negative control (n = 40), the second as a positive control (n = 20) and the remaining three (n= 20 per condition) were exposed the 0.80 µM Cu and 0.25 µM Cd and a combination of these was used for the mixed exposure. Exposure was conducted for a period of 96 hpf (hours post fertilisation) and the whole experiment was repeated twice. Unfertilized eggs, as well as the eggs with irregularities and/or injuries were discarded. The solutions were

renewed with fresh solutions after 48 hours. All experiments were performed using OECD test guideline number 236 (OECD TG 236). For metal accumulation and gene expression analysis, embryos from all treatments were sampled at 96 hpf, washed with medium and placed in 1.5 mL polypropylene centrifuge tubes. The remaining water was removed from each sample with a pipette and snap frozen in liquid nitrogen. The samples for metal analysis were weighed and stored at -70 ^{0}C until use, whereas the samples for gene expression analysis were stored in RNAlater (RNAlater® solution, Ambion, Lithuania) overnight at 4 ^{0}C and then at -70 ^{0}C until further investigation.

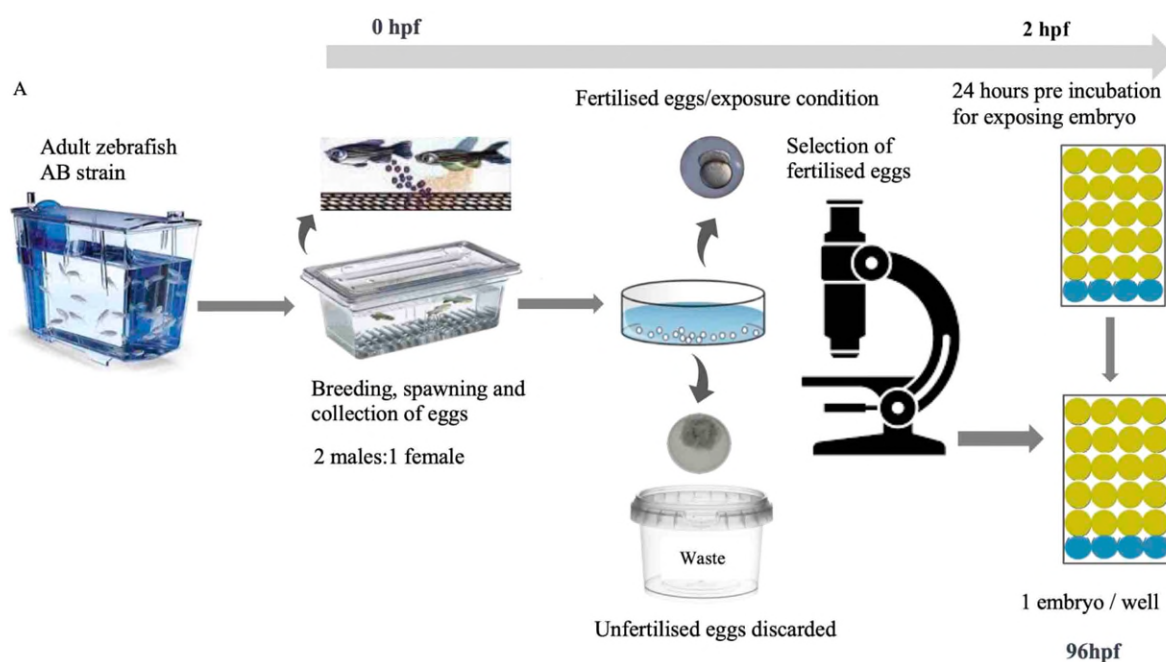

Fig. 1. Illustration of early life stage zebrafish acute toxicity test (from left to right): breeding, spawning, collection of eggs, selection of eggs using an inverted microscope and distribution of embryos over pre incubated 24 well plates prepared with respective exposure concentrations/control. n=20/exposure condition + 4 internal negative control / plate; hpf = hours post fertilisation.

2.2.2 Zebrafish adults

Adult zebrafish (*Danio rerio;* average fresh weight: 0.45 ± 0.10 grams, average length: 2.0 ± 0.04 cm) obtained from the University of Antwerp zebrafish facility were acclimated to EPA medium hard water ($NaHCO_3$: 96 mg/L, $CaSO_4.2H_2O$: 60 mg/L, $MgSO_4.7H_2O$: 60 mg/L, KCl: 4 mg/L; hardness: 80–100 mg/L $CaCO_3$) in a well-aerated 100 L glass aquarium over a 14-day period. After acclimation, fish were weighed and randomly transferred to polypropylene aquaria containing 7.5 L of exposure medium. Each aquarium comprised 5 fish and each

exposure were run in duplicate to obtain 10 individuals per treatment and keep the number of fish used to a minimum. In these experiments adult zebrafish from both control and treatment groups were sampled after 7 days, weighed, and euthanised by rapid cooling in melting ice (≤ 4 ^{0}C) . To sample the organs, the fish were dissected on ice. The gills, liver, gut, and carcasses were collected entirely and divided in subsamples for metal and molecular analysis. The samples for metal analysis were weighed and stored at -70 ^{0}C until use, whereas the samples for gene expression analysis were stored in RNAlater (RNAlater® solution, Ambion, Lithuania.) overnight at 4 ^{0}C and then at -70 ^{0}C until further investigation. All surfaces and instruments used in the RNA extraction protocol were treated with RNase away (MBP, inc. San Diego, CA) to minimize the contamination of RNA by RNase and DNA which might be present on the surfaces.

Throughout the experiments, the animals were kept under a controlled temperature of 28 ^{0}C and a 14 h light/10h dark regime. The water was continuously aerated with an aeration line. The exposure was semi-static, and solutions were 80% renewed after every 48h with fresh solutions. Concentrations of Cu and Cd were measured on water samples taken before and after water renewal. Briefly, water samples from each tank were collected, filtered using an Acrodisc® 0.20 µm Supor Membrane syringe filter (Pall Life Sciences, Ville St. Laurent, QC) filter, acidified with 200 µL of ultra-pure nitric acid and analysed for dissolved Cu and Cd by inductively coupled plasma-mass spectrometry (7700x ICP-MS, Agilent Technologies). Throughout the exposure period, the measured Cu and Cd concentrations were 94 – 100% of the nominal concentrations. The concentrations of these metals in the control medium were below the method quantification limit (< 0.1µg/L). Ammonia, nitrite, and nitrate levels were measured every 24 hours using Tetratest (Tetra®, Melle, Germany) and maintained within the optimal range for zebrafish (ammonia <0.02 mg/L; nitrite < 0.1 mg/L; nitrate < 50 mg/L; Avdesh et al., 2012; Pilehvar et al., 2020). During the acclimation period, fish were fed once daily with commercially available food (Sera, Vipan®, Germany), corresponding to approximately 1% of fresh body weight. No food was given during the exposure phase to avoid ambiguity in the uptake pathways as well as to prevent increased levels of ammonia production.

2.3 Selection of exposure concentration

In an initial series of experiments, the acute 10 days toxicity of Cu and Cd in adult zebrafish was determined by constructing dose-response relationships and obtain an estimate of the EC_{10} and EC_{50} values for the effects of the individual metals and their combination (based on results included in Pilehvar et al., 2019, 2020). Exposure concentrations were selected based on the partial factorial design with total Cu concentrations in the range *ca*. of 0.1 to 8 μM, and Cd concentrations in the range *ca.* 0.2 to 22 μM. The acute toxicity of Cu in adult zebrafish was much higher compared to Cd with respective LC_{50}/LC_{10} values of 216/91 μg/L (3.40/1.43 μM) and >2500/1750 μg/l (22.2/15.5 μM) in medium hard water. However, in combined Cu/Cd scenarios, the toxicity of the mixture becomes much stronger, indicative of a strong Cu/Cd toxicological interaction.

2.4 Morphological, physiological and survival analysis in embryos

A teratogenicity test based on OECD guidelines (OECD, TG 236) was conducted in embryos to assess the effect of metal exposure on embryonic development. Various morphological and physiological changes, as well as the survival was assessed. Exposures of developing embryos were started 0 – 2 hpf. Briefly, embryos were randomly distributed over 24 well plates (saturated overnight with the test solutions) with one embryo per well containing 2 ml of solution (20 treatment and 4 internal negative controls). Two replicate plates were prepared for each treatment including the negative controls. In addition, two plates with 4mg/L 3,4 – dichloroaniline (CAS 95 – 76 – 1, 98% purity, Sigma – Aldrich) were also used as positive control (OECD TG 236) to compare the sensitivity of embryos between different exposure groups. All plates were covered with Parafilm (Parafilm® M, Bemis Europe, Soignies, Belgium). The embryos were kept in an incubator (MIR-254-PE, Panasonic, TCPS, Rotselaar, Belgium) at a constant temperature of 28.5 ± 0.2 ◦C and a 14/10 h light/dark cycle. The solutions were renewed with fresh solutions every 48 hours. Mortality and hatching were checked every day, and a full morphological scoring was performed at 96 hpf. using a Leica S8APO stereomicroscope (Leica Microsystems GmbH, Germany) until 96 hpf (OECD TG 236). Dead embryos as well as the remaining chorion after hatching were removed every 24 hours. Distinct lethal (non-detachment of tail, absence of somites, absence of heartbeat, lack of hatching), sublethal (impaired eye development, deviating pigmentation, oedema, blood accumulation) and morphological malformations (head and tail malformations) were observed and recorded. To assign the presence of the abnormalities, all the parameters

were scored either as 0 if normal and 1 if abnormal. For assessing mortality four endpoints, namely, lack of somites, lack of detachment of the tail, coagulation and absence of heartbeat were checked every 24h.

2.5 Behaviour analysis in embryos

The embryos used for teratogenicity analysis, were also examined for behavioral effects. To evaluate behaviour, the locomotor activity including spontaneous movements and the swimming activity were monitored. Starting from 24 hpf, spontaneous tail movements were visually monitored for a 1-minute time frame in each embryo using the stereomicroscope. The swimming activity was determined in 24-well plates using a Zebrabox video tracking device (Viewpoint, Lyon, France) after 96 hours of exposure. Before analysis, the embryos were allowed to acclimate for 20 minutes to the Zebrabox environment. After the acclimation period, the movement of each embryo (1 embryo/well), was recorded for 40 minutes in 100% light (1200 lux). From the video track data, the locomotor behaviour activity (swimming speed, duration of swimming, and distance travelled) was determined. Data were analysed with the ZebraLab software version 3.20.5.104. In total 40 embryos from two replicates of each of the exposed and unexposed group were used for analysis.

2.6 Morphological, physiological and survival analysis in adults

Morphological changes, including abnormal skin pigmentation and physiological abnormalities such as loss of equilibrium, abnormal swimming behavior, ventilatory function and other visible abnormalities (as clinical signs) were visually monitored to detect deviations from normal body form and overall health, following OECD guidelines (OECD Test No: 203). Observations were recorded at 24 hours interval throughout 7 day exposure. Simultaneously, mortality was recorded every 12 hours to assess effect on survival across all the exposure groups. Fish were considered dead if no visible movement (e.g. gill movements) and no reaction upon touching the caudal peduncle were seen (OECD Test No: 203).

2.7 Behaviour analysis in adults

Visible abnormalities related to behavior were assessed to detect any deviation from normal behaviour and were conducted in accordance with OECD guidelines (OECD Test No: 203). Changes in equilibrium such as abnormal horizontal and vertical orientation as well as loss of buoyancy were monitored and recorded at the interval of 24 hours. In addition, abnormal

swimming behaviour including hypo/hyperactivity, corkscrew swimming, convulsions, tetany, over/under reactivity to stimulus was monitored.

2.8 Metal accumulation in embryos and adults

The accumulation of Cu and Cd was determined by inductively coupled mass spectrophotometry (ICP-MS) in both larvae and adults. In addition, the concentration of essential metal ions, including sodium (Na), magnesium (Mg), potassium (K), calcium (Ca), iron (Fe) and zinc (Zn) were measured in all tissue samples, to determine any alterations in their levels, considering their physiological significance and their potential interactions with Cu and Cd (Mebane, 2023; Liu et al., 2023). For the metal analysis, the larval samples were the same ones used for teratogenicity analysis and the adult samples were derived from those for gene expression analysis. The tissue samples (10 samples per condition) were thawed, weighted, and dried at 60 °C for 24 hrs. After drying the tissues were digested with 0.5 mL ultra-pure nitric acid (Merck, Darmstadt, Germany) in a microwave at 100 watts (3-minutes x 3 times), 180 watts (3-minutes x 3 times), 300 watts (1-minute x 1 time). Digestates were then diluted up to 4 mL with ultra-pure water (Milli-Q, Bedford, MA, USA). An ICP-MS (7700x ICP-MS, Agilent Technologies) was used to analyze the accumulation of Cu and Cd. Reference standard mussel tissue (NIST - 2976) and blank solutions were also prepared for comparison and testing the validity of the method. Fish carcasses were digested in 50 ml tubes at 110 °C on a Hot Block (Environmental Express) for 3 hours (hot block CAL 3300, Environmental Express, USA). Digestates were then diluted up to 30 mL with ultra-pure water (Milli-Q, Bedford, MA, USA). An ICP-MS (7700x ICP-MS, Agilent Technologies) was used to analyze the accumulation of metals. Triplicate samples of a reference standard mussel tissue (NIST-2976) and reagent blank solutions were prepared for method validation. Water samples (10 mL) were collected from each aquarium after every 24 h period, filtered through 0.20 µM filter, acidified with 200 µL of ultra-pure nitric acid and stored for dissolved metal concentration measurements.

2.9 Gene expression in embryos and adults

2.9.1 Primer design

Candidate genes were selected based on their biological, physiological or functional relevance to metal toxicity. Primers of all selected genes for zebrafish were designed from the nucleotide sequences given on NCBI (http://www.ncbi.nlm.nih.gov/Genebank). To

optimize the design of primers across exon boundaries, primers were initially manually designed for each gene, and these were subsequently analyzed using the primer analysis software LightCycler® probe design software (version 3.5, Roche Molecular Biochemicals, Germany). All the primer sequences are listed in Table 1 and 2.

2.9.2 RNA extraction and cDNA synthesis

RNA was extracted using a phenol-chloroform extraction procedure (Chomczynski and Sacchi, 2006n). Snap frozen embryos and adult zebrafish samples were dissolved in 200µL lysis buffer (Qiagen, catalogue number 79216) containing 1% ß-mercaptoethanol and precipitated with Na-acetate and 70% ethanol. The concentration and purity of RNA was determined spectrophotometrically using a Nanodrop ND-1000 spectrophotometer (NanoDrop® ND-1000, ISOGEN Life Science). Genomic DNA was removed with the Turbo DNA free kit (Ambion® Thermo Fisher Scientific). cDNA was synthesized using Superscript TM III first-strand synthesis supermix (Thermo-Fisher Scientific) according to the manufacturer's instructions. cDNA samples were stored at -20 °C until their use for amplification by real-time PCR.

2.9.3 Real – time quantitative PCR (qPCR)

Gene expression analysis using quantitative real-time qPCR was performed in an optical 96 well plate (Applied Biosystems, Thermo Fisher Scientific) using Fast SYBR Green master mix (Applied Biosystems, Thermo Fisher Scientific) amplified and detected by using the 7500 Fast Real-time PCR System (Applied Biosystems, Life Technologies). Primer efficiencies calculated as $E=10^{-1}$/slope was evaluated by a four-point standard curve, prepared by a 1:3 serial dilution of cDNA. Selection of potential reference genes for the normalisation of qPCR data was based on the method given by Vandesompele et al. (2002) and Tang et al. (2007). From the most used reference genes that were tested (see Table 1), the at least three (in adults depending upon tissue) or four (in embryos) most stable reference genes were selected by GeNorm analysis. Gene expression analyses were performed, in accordance with the MIQE guidelines (Minimum Information for Publication of Quantitative Real-Time Experiments) (Bustin et al., 2009) and the technique as described by Rocha et al., (2016). Gene expression (transcriptional) profiles of the genes related to the cellular redox state, cell cycle and (DNA) repair mechanisms, structural development and metal transport were studied (see Table 2). Relative quantification of each gene expression level was normalized according to the

expression of most stable reference genes. The details of the procedure are given in Table S1 (see supplementary data).

2.10 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8.1.2 statistical software (GraphPad software, CA, USA). In all experiments, each parameter was analyzed separately in comparison with the appropriate controls. Data were tested for normality of distribution using the D'Agostino-Pearson test. Based on normality distribution, the results were either analysed by parametric test (one way ANOVA), when assumptions of normality were met and/or non-parametric test (Kruskal – Wallis one way ANOVA), when assumptions were not met. For parametric test, Holm-Sidak's multiple comparison test was used to compare the treatment and control group and for non-parametric test Dunn's method was used for multiple comparison. A p-value < 0.05 were considered statistically significant.

Table 1. Nucleotide sequence of the specific primer pair of reference genes for zebrafish *(Danio rerio)* used in the study.

Gene	Function	Accession number	5' – 3' Sequence
beta actin	Cytoskeleton protein	NM181601	F: ACTGTATTGTCTGGTGGTAC R: TACTCCTGCTTGCTAATCC
β2m	Beta chain of major histocompatibility complex I molecule	NM131163.2	F: CCAAAGTAGCTGCTACAGG R: GCCAACAAGTGCAGAGT
ef1α	Protein translation	ENSDART0000 0023156	F: CTGGAGGCCAGCTCAAACAT R:ATCAAGAAGAGTAGTACCGCTAGCATTAC
sdha	Oxidative phosphorylation	NM200910.1	F: GTCCTATGTGGATCCCGA R: GATTGCAGGAGGAATGGC
rnap	Transcription	AY648795.1	F: TTCAGCCGCTTCAAGAAC R: CTGCTTCAGGACACAGAT

Table 2. Nucleotide sequences of the specific primer pair of genes of interest for zebrafish *(Danio rerio)* used in the study.

Gene	Function	Accession number	5' – 3' Sequence
cat	Oxidative stress	NM130912.2	F: CAGACAAGATGCTGCAGG R: CTGATAGTTTGCCACACG
gsr	Oxidative stress	NM001020554	F: ACAGTCAGTGAGGATGATGTGCCAG R: TAGACCCAAGAGTGGAAAGAATACCAGC
gstm	Oxidative stress	NM001162851	F: GCTGGGGACAAGATCACATT R: TTTGGCCATCTTGTTGTTCA
gpx	Oxidative stress	AW232474	F: AGATGTCATTCCTGCACACG R: AAGGAGAAGCTTCCTCAGCC
sod1	Oxidative stress	NM131294.1	F: GTTTCCACGTCCATGCTT R: CTCACACTATCGGTTGGC
sod2	Oxidative stress	NM199976.1	F: AGATTGAGGATTGCAGCG R: CGCATGTTCCCAGACATCTA
sod3	Oxidative stress	NM001099236.1	F: TCCCGGAGATATGGGCAAC R: GGACTGACCTCCAAACAGC
hsp70	Heat shock	AB062116.1	F: GCCAACGGGATCCTAAATG R: TCTCCTCTTTGCTCAGCC
hsp90	Heat shock	NM131328.1	F: TGAGGATCTGCCTCTGAAC R: TCGGTGAAGAGATCGAGAC
p53	Apoptosis	AF365873	F: GGGCAATCAGCGAGCAAA R: ACTGACCTTCCTGAGTCTCCA
bax	Apoptosis	NM 131562	F: CAGGGATGCTGAAGTGACCC R: ACAAGGCGACAGGCAAAGTA
bcl2	Apoptosis	NM001030253.2	F: TGGCGTCCCAGGTAGATA R: CGTACATCTCCACGAAGG
casp3	Apoptosis	NM131877	F: CCGCTGCCCATCACTA R: ATCCTTTCACGACCATCT
casp9	Apoptosis	NM152884	F: AAATACATAGCAAGGCAACC R: CACAGGGAATCAAGAAAGG
gadd45	DNA repair	NM213031.3	F: GCATGGTACATTCCACCC R: GCTCATGTTCCCACAACT
rad51	DNA repair	NM213206.2	F: CCAAGAAGCCTATTGGTGG R: TCTGGTAAACACGGAGAGTC
hhex	Embryonic development	NM 130934	F: GGTAAGCCTCTGCTGTGGTC R: TCTTCTCCAGCTCGATGGTT
pax8	Embryonic development	AF072549	F: GAAGATCGCGGAGTACAAGC R: CTGCACTTTAGTACGGATGA
ngn1	Embryonic (neuro) development	AF017301	F: ATTCTGCAAAACCTCAAGCATCTC R: TGTACACTACGTCGGTTTGCAAGT
neuro D	Embryonic (neuro) development	AF017302	F: AACGATATGGAAGACGACGATGAT R: GCATGGTAAACGCGTAGTTCTTCT
shha	Embryonic (neuro) development	NM 131063.1	F: AGACCGAGACTCCACGACGC R: TGCAGTCACTGGTGCGAACG
mt	Metal transport	NM001131053	F: TGTGCCAAGACTGGAACTTG R: CTTCATTGACAGCAGCTGGA
ctr1	Metal transport	NM001320405.1	F: TTGCGGAGTTTGAAGTCG R: GGTGACACTGGCATCAGATA
ecac	Metal transport	NM001001849.1	F: GGACCACACACTCTTTACC R: GTGTCACTCATCATGGCGA
zip1	Metal transport	NM212583.2	F: TCCTTCATGCTCTTCCTGTC R: TCTCCAACACCTTGGCATTC

3. Results

3.1 Assessment of Metal Effects in embryos

Zebrafish embryos exposed to the control solution (culture medium) showed normal development, whereas, all the metal-exposed embryos showed different signs of toxicity. While some of the alterations were lethal, such as lack of hatching and survival, others were

non-lethal and/or teratogenic within the exposure period, such as morphological and physiological symptoms including oedema, blood accumulation (blood clogging), impaired eye development, head and tail malformations (Fig. 2 and Table. 3).

Zebrafish embryo hatching begins around 48 hpf with complete hatching around 72hpf (Westerfield, 2000). We examined hatching at 48, 72 and 96 hpf. Compared to the control group, no hatching was observed in 80% of embryos exposed to Cu alone and in 95% of embryos exposed to Cu+Cd at 96 hpf. Exposure to Cd alone induced a delay in the hatching period of approximately 30% embryos at 72 hpf, while at the same time unexposed embryos showed 100% hatching success. At 96 hpf, 10% of Cu alone and 40% of Cu+Cd exposed embryos died within the chorionic membrane, apparent by the absence of heartbeat and/or coagulation of mass, at the same time 5% of Cd exposed embryos died. DCA (3,4 – dichloroaniline), used as a positive control, induced 70% mortality at 72h, increasing to 100% at 96 hpf. Regarding the morphological and physiological symptoms, several alterations were observed. In general, all metal exposures induced oedema in approximately 80% of Cu+Cd, 60%, Cu and 5%, Cd exposed animals, including both hatched and/or unhatched animals. Another common effect of metal toxicity was tail malformation, which mainly involved curvature of spine. From the total number of embryos hatched in each metal-exposed group, the curvature of spine was observed in the order of Cu+Cd (60%) > Cu (20%) > Cd (5%). In approximately 20% Cu alone, 5 % Cd alone and 30% Cu+Cd exposed embryos accumulation (clogging) of blood was observed in the tail region. A notable effect observed in 5% of Cu+Cd exposed embryos was the impaired eye development and malformed head, this effect was not observed in the other treatments (see Table 3).

Behaviour was assessed by examining the spontaneous movements of the embryos inside the chorion and the swimming ability of the larva after 96 hpf days. Spontaneous movements were absent in approximately 60% (22 of 36 surviving embryos) of the Cu-exposed and in 70% (17 of 24 surviving embryos) of the Cu+Cd-exposed embryos, whereas Cd-exposed embryos and the control group showed normal movements. No swimming was observed in Cu+Cd-exposed embryos whereas, Cu alone and Cd-exposed embryos, were negligibly affected (Fig. 3).

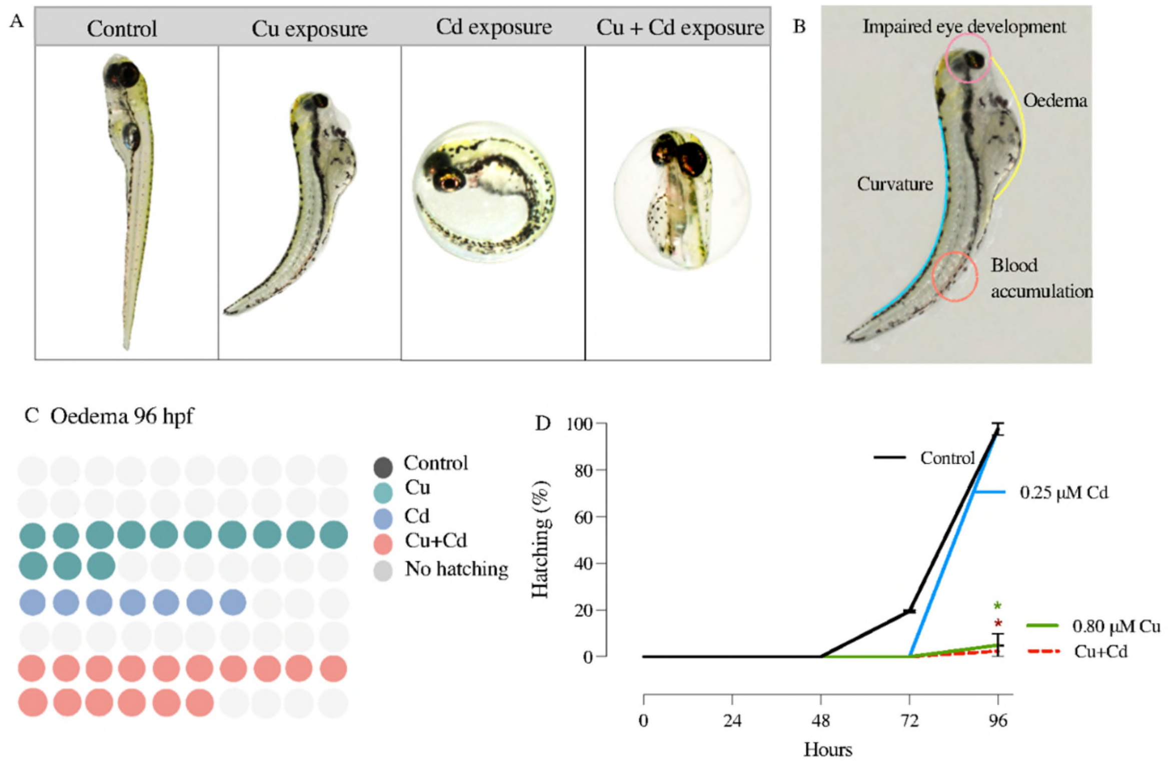

Fig. 2. Morphology and Development of zebrafish embryos. (A) Microscopic images of teratogenic effects in zebrafish embryos exposed to 0 µM (control), 0.80 µM Cu, 0.25 µM Cd and the mixture at 96 hpf. (B) Illustration of malformations in hatched zebrafish embryos (impaired eye development, tail curvature, oedema, blood clogging). (C) Graphical representation of embryos oedema at 96 hpf. (D) Graphical representation of hatching rate of zebrafish embryos in exposed and control groups at different time points. The data points represent average ± standard error of mean (SEM) of minimum 20 biological replicates. Two independent experiments were performed and the results of two experiments confirm each other. *p<0.05.

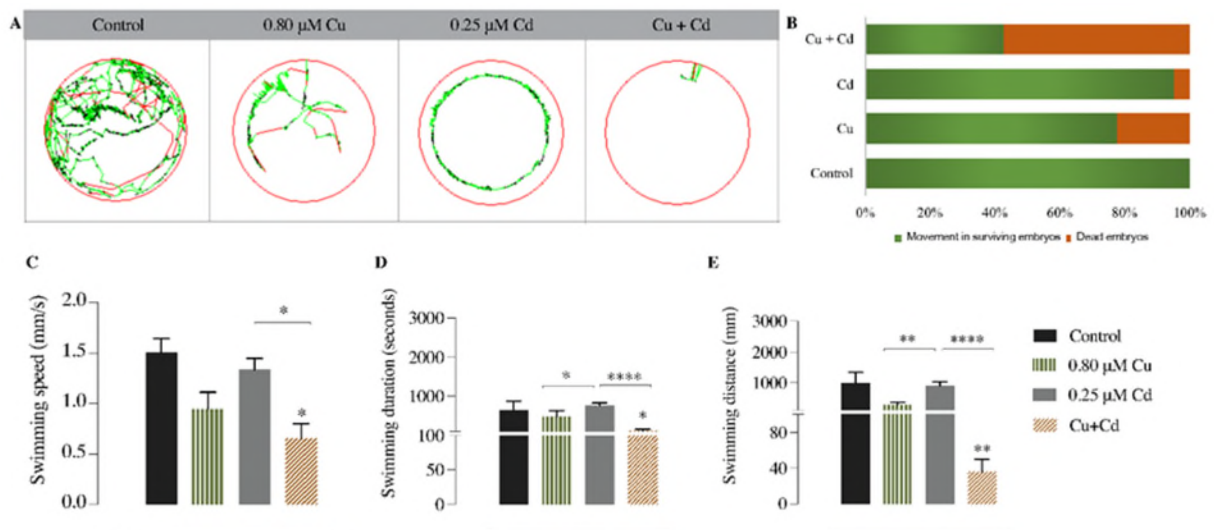

Fig. 3. **Behavioral analysis of zebrafish larvae**: (A) Representative video tracks of zebrafish larvae exposed to 0µM (control), 0.80µM Cu, 0.25µM Cd and Cu+Cd at 96hpf. (B) Movements in surviving embryos (C) swimming speed (mm/s), (D) swimming duration (seconds) and (E) swimming distance (mm). The data points represent average ± standard error of mean (SEM) of minimum 20 biological replicates. Two independent experiments were performed and the results of two experiments confirm each other. *$*p<0.05$, $**p<0.01$, $****p<0.0001$*

Table 3. Overview of lethal, sublethal and teratogenic effects of 0.0 µM (control) 0.80 µM Cu, 0.25 µM Cd and Cu+Cd co-exposure in zebrafish embryos

Toxicological endpoints	Negative control				Cu				Cd				Cu + Cd			
	Exposure time in (h)															
	24	48	72	96	24	48	72	96	24	48	72	96	24	48	72	96
Lethal endpoints																
(Mortality: OECD																
TG 236)	0	0	0	0	0	0	0	3	0	0	0	2	0	0	0	10
Coagulation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lack of tail		0	0	0		0	0	0		0	0	0		0	0	0
detachment		0	0	0		0	0	7		0	0	3		0	10	30
Lack of somite																
formation																
Lack of heartbeat																
Sublethal																
Lack of hatching		0	0	0		100	80	80		30	30	0		100	95	95
Impaired eye		0	0	0		0	0	0		0	0	0		0	0	5
development		0	0	0		0	0	0		0	0	0		0	0	0
Deviating		0	0	0		0	0	60		0	0	5		0	0	80
pigmentation		0	0	0		0	0	20		0	0	5		0	0	30
Oedema		0	0	0		0	0	0		0	0	0		0	0	5
Blood		0	0	0		0	20	20		0	5	5		0	60	60
accumulation																
Head																
malformation																
Tail malformation																

Effects are given in percentage of animals affected in each exposure group. Percentages for sublethal endpoints are given as percentage of surviving embryos.
Oedema was observed as a distinguishable or non – distinguishable bulge in pericardium and/or yolk sac.
Tail malformation was observed as a bent or curved tail.

3.2 Assessment of Metal Effects in adults

The animals exposed to Cu and Cd singly showed no signs of morphological or physiological or any other alterations, whereas, 20% animals exposed to the mixture exhibited hypoactivity and mild signs of oedema (abdominal swelling). Oedema was observed in the

same individuals which showed hypoactivity. The results are summarised in Table 4. Furthermore, there was no effect on the survival of adult zebrafish across all experimental conditions.

Table 4. Overview of various endpoints assessed in zebrafish adults after exposure to 0.0 µM (control) 0.80 µM Cu, 0.25 µM Cd and Cu+Cd co-exposure for 7-days.

End point (OECD TG 203)	Sub-categories	Control	CU	Cd	Cu + Cd
Loss of equilibrium	Abnormal horizontal orientation	-	-	-	-
	Abnormal vertical orientation	-	-	-	-
	Loss of buoyancy control [1]	-	-	-	-
Abnormal swimming behaviour	Hypoactivity	-	-	-	20
	Hyperactivity	-	-	-	-
	Corkscrew swimming [2]	-	-	-	-
	Tetany [3]	-	-	-	-
	Over active to stimulus	-	-	-	-
	Under active to stimulus	-	-	-	-
Abnormal ventilatory function	Hyperventilation [4]	-	-	-	-
	Hypoventilation [4]	-	-	-	-
Abnormal skin pigmentation	Darkened	-	-	-	-
	Lightened	-	-	-	-
	Mottled	-	-	-	-
Other visible abnormalities	Exophthalmia [5]	-	-	-	-
	Oedema [6]	-	-	-	20
	Hemorrhage [7]	-	-	-	-
	Mucus secretion	-	-	-	-
	Faecal casts	-	-	-	-
	Aggression	-	-	-	-

1 : Floating at surface or sinking to the bottom
2 : Rotation around long axis; erratic movements
3 : Rigid body musculature (intermittent or permanent)
4 : Increased / decreased frequency of opercular ventilatory movements
5 : Swelling within orbital socket(s) resulting in bulging of one or both eyes
6 : Abdominal swelling due to accumulation of fluid
7 : Blood spots due to intradermal or sub-mucus bleeding
Minus (-) sign indicates "no abnormalities were observed"
Effects are given in percentage of animals affected.

3.3 Metal accumulation in embryos and adults

Accumulation trends of Cu, Cd, iron (Fe), zinc (Zn), sodium (Na), potassium (K), calcium (Ca), and magnesium (Mg) were determined after the embryos and adults were exposed to Cu and Cd alone and in the presence of the two metals. In embryos, Cu levels remained similar following Cu exposures in single exposure and in combination with Cd. Cadmium levels were increased in embryos in single exposures followed by embryos in combined exposures (Fig. 4A). Analysis of other essential metals revealed a significant decrease in the concentration of Na and Ca in the embryos exposed to both Cd and Cu simultaneously as compared to the unexposed condition (see Table S2 in supplementary data).

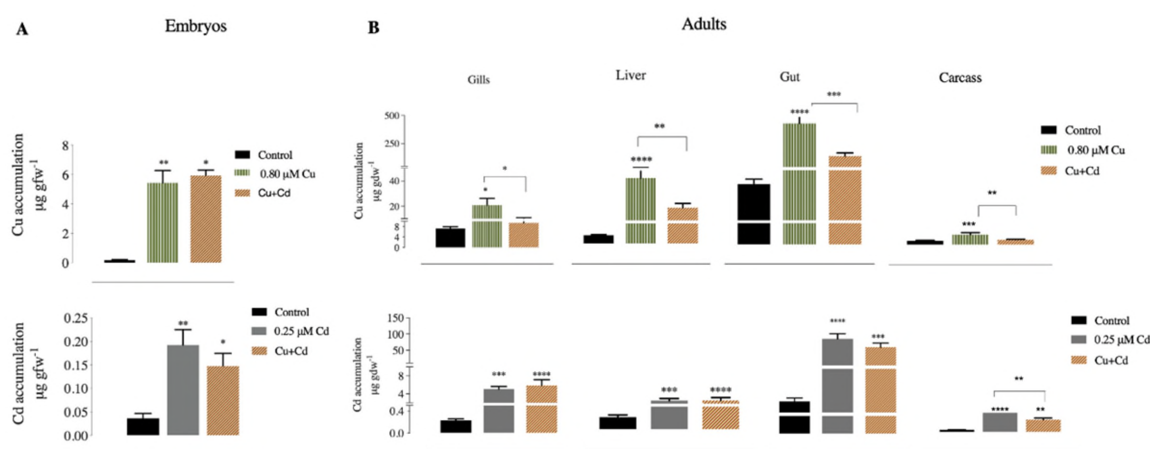

Fig. 4. Metal accumulation. (A) Cu and Cd accumulation in zebrafish embryos 96 hpf exposed to 0.80 µM Cu, 0.25 µM Cd and Cu+Cd. The values are the average of ± standard error of mean (SEM) of minimum ten biological replicates in each exposure group. (B) Cu and Cd concentration in zebrafish adults exposed to 0.80 µM Cu, 0.25 µM Cd and Cu+Cd. The values are the average ± standard error of mean (SEM) of minimum ten biological replicates in each exposure group All the metal present in the experimental media were in the dissolved phase and the total measured metal concentration was 94 – 100% of the desired nominal concentrations. Two independent experiments were performed and the results of two experiments confirm each other. *$*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$.*

In adults, the concentrations of Cu, Cd, Fe, Zn, Na, K, Ca, and Mg were determined in different organs, i.e., the gill, liver, gut, and the carcasses (which include the muscle tissue and bones) and are shown in Fig. 4B (Cu and Cd) and table S3 (see supplementary data). The exposure to Cu and Cd lead to accumulation in all the tissues. Cu concentrations increased significantly in all tissues when the animals were exposed to Cu alone. In the animals exposed

to a combination of Cd and Cu, the Cu levels increased as compared to the unexposed group but were lower compared to Cu alone. Increases in Cd were similar in both Cd exposure conditions, irrespective of the tissues involved. In addition, increased levels of Ca and Mg were observed in Cu exposed gills and in Cu+Cd exposed liver tissue. Also, Zn concentrations were significantly increased in Cu+Cd exposed liver, but not in the single Cu or Cd exposures.

3.4 Gene expression analysis in embryos and adults

In embryos (Fig. 5), the antioxidant genes, including glutathione reductase (*gsr),* glutathione transferase *(gstm)* and superoxide dismutase *(sod1),* exhibited a significant decrease in the Cu+Cd mixture group, while no significant effect was seen in single exposure conditions. Exposure to Cu alone and Cu+Cd led to an upregulation of the DNA repair gene (*gadd45ba)* and the calcium transport gene (*ecac)*. These genes remained unaltered in the Cd exposed group. In contrast to the other conditions, exposure to Cu alone led to an upregulation of the apoptosis-related gene *bcl2.* Notably, the activity of the heat shock genes *(hsp70, hsp90),* the neuronal gene *(neuroD)* and the metal transport gene metallothionein (mt) was altered under all exposure conditions. Metallothionein and *ecac* were upregulated, while, *neuro D* was downregulated.

In adults (Fig. 6), exposure to Cu and Cd resulted in the upregulation of *ecac* in gills, which remained unchanged in Cu+Cd exposure group. The exposure to Cu and Cd mixture resulted in alterations in the activity of more number of genes. Specifically, the antioxidant genes, *gsr* and *sod1* showed upregulation, while *sod2* and *sod3* showed downregulation. In addition, upregulation of the pro-apoptotic gene *bax* and a downregulation of anti-apoptotic gene *bcl2* was observed under the mixture condition. Genes involved in DNA repair exhibited varying expression: *rad51* was upregulated, while *gadd45* was downregulated. In addition, the heat shock gene *hsp70* and the copper transporter gene *ctr1* were upregulated. Notably, *mt* was upregulated in all the three exposure groups.

In the liver, changes in gene expression were only evident in response to the Cu+Cd mixture. In particular, the antioxidant genes, including catalase (*cat*), *sod1* and *sod2,* exhibited upregulation. In addition, the Cu+Cd mixture induced upregulation of *hsp70, mt, ctr1* and the zinc transport protein (*zip1).* At the same time, a downregulation of *ecac* was observed in Cu+Cd exposed group. Notably, in liver the activity of DNA repair genes matched with

those of gills, showing *rad51* was upregulated, while *gadd45* was downregulated In gut, three genes, *cat, hsp70, and mt* were upregulated in both single and combined exposure group. Exposure to Cd also led to upregulation of another antioxidant gene (*gsr)* whereas, Cu+Cd led to up-regulation of *sod3* and apoptotic genes *bcl2* and downregulation of *bax.* In gut, the activity of DNA repair genes were similar to gills and liver. The results depicting metal accumulation and gene expression patterns in both embryos and adult organisms are comprehensively illustrated in Figure 7 for the comparative analysis.

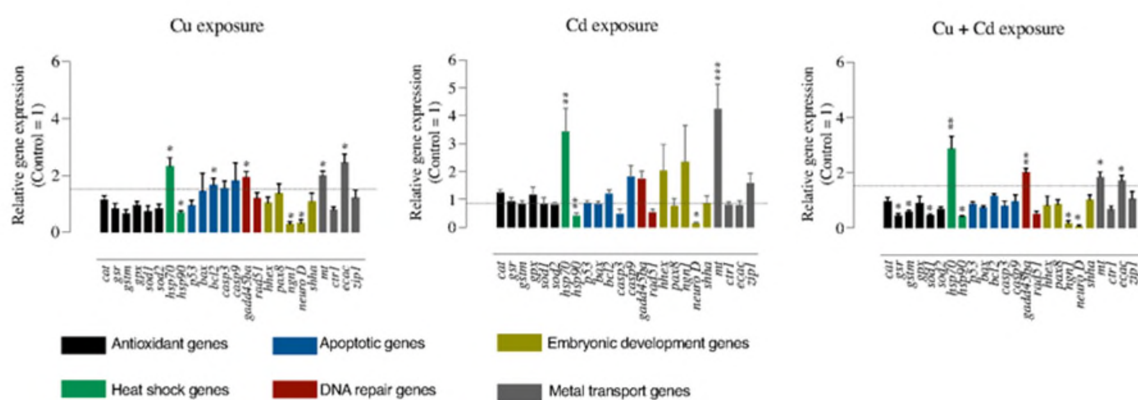

Fig. 5. Gene expression analysis of Zebrafish embryo. Relative gene expression levels of genes representing different classes of genes (antioxidative, apoptosis, DNA repair, development, and metal transport) in zebrafish embryos exposed to 0.80 µM Cu, 0.25 µM Cd and Cu+Cd at 96 hpf. The values are the average ± standard error of mean (SEM) of minimum six biological replicates. Two independent experiments were performed and the results of two experiments confirm each other. **p<0.05, **p<0.01, ***p<0.001.*

4. Discussion

A clear understanding of the adverse effects of environmental stressors on developing organisms is crucial to assess their short- and long-term impacts. During development, toxicants can interfere with essential molecular components in different phases and life stages (Jezierska et al., 2008; Little et al., 2014; Takeuchi et al., 2016; Majid et al., 2022), and a proper mechanistic understanding at different biological levels is needed. In this perspective, the present study was designed to understand the mechanisms of toxicity in developing zebrafish embryos and adults after exposure to Cu and Cd separately and as a binary mixture.

Acute developmental toxicity in zebrafish embryos was assessed by monitoring sub-lethal and lethal endpoints. Copper exposure induced various physiological and morpho-physiological effects, including impaired hatching, oedema in the primary organ systems (pericardium and yolk sac), blood accumulation and tail malformations (Fig. 2, Table 3). In particular, hatching was strongly impaired in the embryos exposed to Cu, both individually and in combination with Cd, with embryos being partially or completely entrapped in the chorion. In contrast, Cd exposure only led to a delay in hatching. Hatching is a crucial event during embryogenesis, in which the embryo emerges from the chorion and begins to move freely. Despite the protective function of the chorion, metals can cross it and adversely affect the development process (Wang et al., 2020). Copper-induced hatching impairments in various fish species, including zebrafish been documented in literature (Jezierska and Witeska, 2001; Johnson et al., 2007; Jezierska et al., 2009; Zhang et al., 2018). Similarly, Cd exposure has been associated with delayed hatching of fish subjected to Cd intoxication.

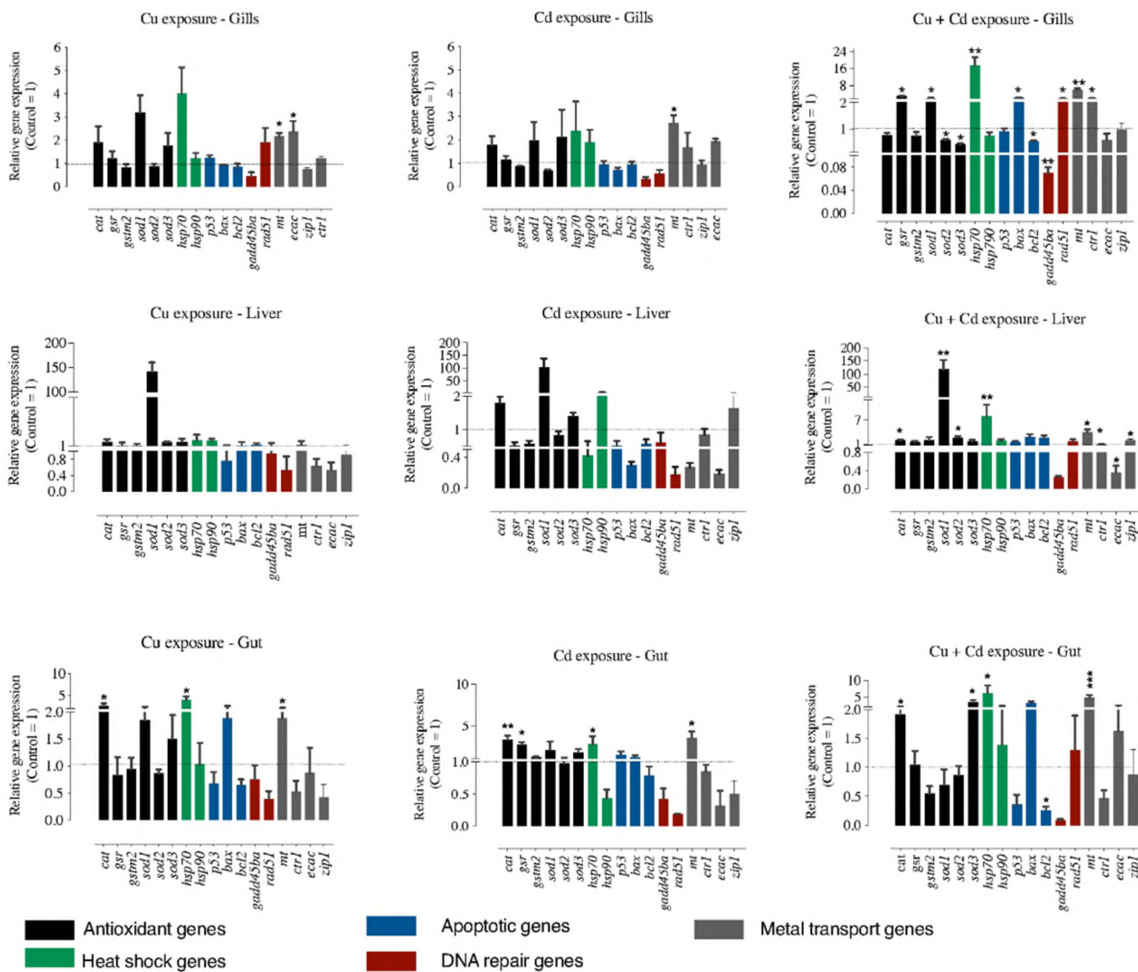

Fig. 6. Gene expression analysis of Zebrafish adults. Graphs representing the relative gene expression levels of genes representing different classes of genes (antioxidative, apoptosis, transport and DNA repair related) in gills, liver and gut of adult zebrafish exposed to 0.80 µM Cu, 0.25 µM Cd and Cu+Cd after 7 days exposure. The values are the average ± standard error of mean (SEM) of minimum six biological replicates in each exposure condition. Two independent experiments were performed and the results of two experiments confirm each other. *$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.*

For example, Aldavood et al. (2020) reported hatching inhibition only at a high Cd concentrations (3.1 µM), whereas Mitovic et al. (2021) observed delayed hatching at a comparatively higher Cd concentration (16.7 µM). Hatching impairment may be associated with biochemical processes, such as a decrease in the synthesis of hatching enzymes by the hatching gland for chorion digestion (Fraysse et al., 2006). While our study did not investigate the influence of metal toxicity on these biochemical pathways, it presents an opportunity for future exploration. In addition, several other factors have been described in literature that cause hatching inhibition, including direct damage to the chorion, oxidative stress, disruption of ion regulatory mechanisms, and interference with signalling pathways involved in embryonic development (Jezierska et al., 2009; Martin et al., 2011; Zhang et al., 2018; Taslima et al., 2022). In a second part of this study, we further focused on the involvement of the redox balance, and compared the induced mechanisms in both embryos and adult fish, to better understand the specific developmental impairments. In doing so, we also evaluated other endpoints, and a potential link with underlying redox-related mechanisms.

Impaired hatching and oedema were observed in all metal-exposed groups, with the highest incidence of oedema in embryos exposed to the mixture, followed by Cu and Cd, with Cd having the lowest incidence. Oedema, characterized by fluid accumulation and visible swelling, is linked to cardiovascular dysfunction and metabolic disturbances. Similar trends in blood accumulation and tail malformations were noted. Tail malformations may result from altered developmental signaling pathways (Wnt signaling) (Kimelman, 2016). Embryos exposed to the mixture showed additional malformations, including impaired eye and head development, and a higher mortality rate, suggesting increased effects from higher exposure levels. The higher incidence of effects in the mixture group likely results from the combined presence of both metals.

Morphological and physiological analyses were also carried out in adult zebrafish, but these were less pronounced compared to embryos (Table 4). In contrast to embryos, where oedema was observed under all exposure conditions, in the adults it only occurred in the mixture group. One possible reason for this difference could be the developmental stage-specific sensitivity to toxicants (Mohammad et al., 2013). Embryos may be more susceptible to certain effects due to incomplete development, a higher cell division rate and suboptimal efficiency of protective mechanisms (Mohammad et al.,2013; Groh et al., 2015; Robinson et al., 2020). In contrast, adult fish possess well-developed physiological mechanisms, such as gills, which enhance their ability to maintain internal stability and to mitigate some of these effects (Kashiwada et al., 2008; Mohammad et al.,2013; Sawant et al., 2015).

Building on previous research highlighting behavioral changes as an indicator of neuronal status (Johnson et al., 2007; Jin et al., 2015; Jarema et al., 2022), as well as studies demonstrating the effects of Cu (Sandahl et al., 2007; McIntyre et al., 2012) and Cd (Faucher et al., 2006; Thomas et al., 2016; Utsav et al., 2021) on fish behaviour, we used behavior as an additional parameter to investigate the effects of single and combined toxicity of Cu and Cd in the two life stages. Hypoactivity was observed in both embryos and adults (Figure 3, Table 4), and was again more pronounced in embryos. While the effect was evident in both Cu and mixture-exposed embryos, in adults it was only observed under mixture condition. These results emphasise the different neuronal effects of metal exposure at different life stages, and the need for targeted studies on the long-term neurodevelopmental effects of mixture toxicity and the underlying mechanisms, tailored to specific developmental stages and different species.

To explain the differences in sensitivity between embryos and adults, and to further examine the relationship between toxicological effects and metal exposure, we analyzed metal concentrations to correlate metal accumulation with observed effects. The similarity of the effects observed in embryos exposed to Cu alone, and in combination with Cd indicates that the effects were primarily driven by Cu, and increased in the presence of Cd. This interpretation is supported by the metal accumulation results, which indicate a suppression of Cd accumulation in the presence of Cu. The exposure to Cu and Cd led to their accumulation in both embryos and adults, however, the dynamics of uptake differed. In embryos, Cd uptake was decreased in the presence of Cu, whereas, the opposite was true for adults in which suppression of Cu uptake by Cd was seen in all organs. While the effects

observed in adults can possibly be attributed to Cd levels, further analysis is needed to confirm this hypothesis. In literature, different inhibitory trends have been described, both in favour of Cd and/or Cu uptake. Komjarova and Blust (2008) found inhibition of Cu uptake by Cd in *Daphnia magna.* Similar findings were reported by Castaldo et al, (2020) in the fish species, *Cyprinus carpio*, whereas, the opposite was found in *Danio rerio* (Komjarova and Blust, 2009a; Komjarova and Bury 2014). Studies have shown the existence of Cu – Cd (synergistic) interactions even at low concentrations (Komjarova and Blust, 2008; Cobbina et al., 2015; Pilehvar et al., 2020). At the molecular level, such interactions between essential and nonessential metals are known to occur through the mechanisms of ionic and molecular mimicry due to the similarity of the physicochemical properties of the elements (Bridges and Zalups 2005). These interactions can lead to significant changes in the apparent effects (Altenburger et al. 2013). For example, Cd can replace essential metal ions, mainly Zn^{2+}, Cu^{2+} and Ca^{2+} in metalloenzymes through ionic mimicry (Brzóska and Moniuszko-Jakoniuk 2001) and affect the binding of -SH groups of biomolecules such as enzymes, proteins and nucleic acids to metal ions. (Zalups, 2000).

In embryos, a significant decrease was observed in the internal concentrations of sodium (Na) in response to a co-exposure of Cu and Cd, as opposed to their levels in the single exposure scenarios (see supplementary data, S2). This finding is consistent with previous studies showing Cu induced decrease in Na influx in fish (Witeska et al., 2013, Delahaut et al., 2020). Adults exhibited organ-specific patterns of the accumulation of essential metal ions. A decrease in Na levels was seen in the gill tissue of adults also and is consistent with the previous studies showing reduction in Na levels in the gills of common carp (Delahaut et al., 2020). This further substantiates our assumption that toxicity is driven by Cu in mixed exposure scenario, because one of the primary target of Cu toxicity is Na dysregulation, by inhibiting the functioning of sodium-potassium ATPase (Na+/K+-ATPase) enzyme (Liao et al., 2023). Copper is known to increase cell permeability, resulting in increased Na ion loss (Liao et al., 2023). In addition to Na, the co-exposure to Cu and Cd also resulted in decreased Ca levels. In litrature, this disruption has been attributed to ability of Cu to disrupt Ca homeostasis through various mechanisms, including direct competition with Ca ions for uptake (Liorti et al., 2016). Similar to embryos, liver showed increased levels of Ca. However, on analyzing the transcriptional activity of calcium transporter *ecac,* a decrease was observed, possibly as a compensatory/negative feedback mechanism triggered by the elevated calcium concentrations (Bagur et al., 2017). In addition to Ca, the levels of Mg and

Zn were also elevated. Increase in the levels of Zn could be directly related to the upregulation of zinc transporter zip1 (see Fig 6).

Subsequent organ-wise comparison revealed the highest concentrations of Cu and Cd in gut tissue under both single and mixture exposure conditions, indicating the role of gut in metal accumulation. Despite higher (single) metal accumulation in single exposure groups, more potent effects were observed in mixed exposure groups for both embryos and adults. Adults had higher metal loads than embryos, but embryos showed greater toxicity. This indicates that metal accumulation does not necessarily predict toxicity; internal metal bioavailability, which involves metals interacting with key physiological sites, is crucial. Regulatory guidelines often focus on external bioavailability, influenced by environmental factors like water hardness, which can decrease metal availability and toxicity. Our study found varying water hardness between adults and embryos, contributing to stronger effects on embryos due to the inverse relationship between water hardness and metal toxicity.

To elucidate the mechanistic underpinnings of the observed metal-induced developmental effects, we conducted a comprehensive analysis of gene expression patterns associated with (oxidative) stress mechanisms in both embryos and the gills, liver, and gut tissues of adult zebrafish (Fig. 5 and 6). In literature, both Cu and Cd are known to cause oxidative stress by directly (Cu) or indirectly (Cd) triggering the production of reactive oxygen species (ROS), which can cause cellular impairment via lipid peroxidation, mitochondrial de-regulation, Ca disbalances and/or protein and DNA damage (Rensing and Grass, 2003; Valko et al., 2005; Woo et al., 2009; Birben et al., 2012; Collin 2019). Our data showed stronger effects on the antioxidative machinery in co-exposed embryos and adults. In embryos, exposure to Cu+Cd led to a downregulation of the antioxidant genes *gsr, gstm*, and *sod1,* which remained unaltered in the single exposure scenarios. While the upregulation of antioxidant genes is a well-known response to oxidative stress (Sablina et al., 2005; Lee et al., 2013; Marengo et al., 2016; Ngo & Duennwald, 2022), the observed downregulation in our study may signify a broader cellular response to oxidative stress. This response could involve compromised protective functions due to alterations in signaling pathways, such as the Nrf2 (nuclear factor erythroid 2-related factor 2) pathway. Although we did not assess the activity of Nrf2 signaling, it is closely linked to the antioxidant system, acting as a transcription factor that regulates the expression of antioxidant and detoxification genes (Zhang et al., 2018; Kim et al., 2019; Endo et al., 2020). Alternatively, the downregulation may represent a cellular

adaptation, with the cell prioritizing other survival mechanisms or metabolic processes which may impact the allocation of resources to antioxidant defense mechanisms (Tzatsos & Tsichlis, 2007). Developmental impairments were also observed during exposure to Cu and Cd individually, suggesting the presence of oxidative stress in single metal exposures, albeit not manifested at the transcriptional level of antioxidant genes. In adults, alterations in the antioxidant genes was observed in all three organs, indicating a common toxicity pathway.

Another common response indicative in both embryos and adults was the upregulation of heat shock proteins *hsps* and *mt. Hsp70s* acts as a molecular chaperones and are expressed by cells in response to various stressors, including protein damage caused by oxidative stress (Mosser et al., 2000; Reeg et al., 2016). *Hsp70* was expressed in all metal exposure groups in embryos. In adults, both these genes were induced in the mixture-exposed group only. The upregulation of *hsp70* suggests its cytoprotective role against oxidative stress, as it plays a crucial role in cellular defense mechanisms (Ikwegbue et al., 2017; Kurashova et al., 2019). Metallothionein is known to serves as an important biomarker of Cd exposure and participates in metabolic processes related to metal homeostasis, detoxification, oxidative stress, and neuroprotection (Vasák and Hasler, 2000; Wang et al., 2014). Therefore, the upregulation of *mt* indicates disturbances in metal homeostasis and/or the redox balance. Taken together, the response of *hsp70* and *mt* indicate a common adaptive response to metal -induced stress across these two life-stages. Furthermore, redox alterations have been linked to adverse neurodevelopmental effects (Wells et al., 2009; Salim, 2017), suggesting that they could be the underlying cause of the neurodevelopmental effects observed in the embryos in our study. This finding aligns with a substantial decrease in the expression of the *neuroD* gene in embryos, which is recognized for its pivotal role in embryonic neurogenesis, and as a critical regulator of neuronal cell differentiation and specification in the developing nervous system (Lee et al., 1995; Miyata et al., 1999; Tutukova et al., 2021).

Oxidative stress resulting from metal exposure has been consistently associated with DNA damage (Liu et al., 2009; Balen et al., 2011; Majid et al., 2022). In our study, DNA damage repair was activated in both embryos and adults. The disparity in DNA damage response between embryos and adults suggests differential susceptibility to metal-induced oxidative stress. One possible reason for this difference could be variations in the developmental

stage and physiological resilience between embryos and adults. Specifically, while the increased activity of DNA damage gene *gadd45ba* was observed in embryos, the opposite trend was observed in adults. Additionally, another DNA damage-related gene, *rad51,* was activated in all three tissues in adults. Moreover, the alterations in apoptotic gene expression were specifically seen in the gills and gut of adults, possibly because both organs are primary organs of metal elimination and major targets of metal toxicity. Taken together, the results suggest that while similar toxicity mechanisms may be activated, the response of genes related to these mechanisms may vary depending on the developmental stage, and eventually influence the final adverse outcome.

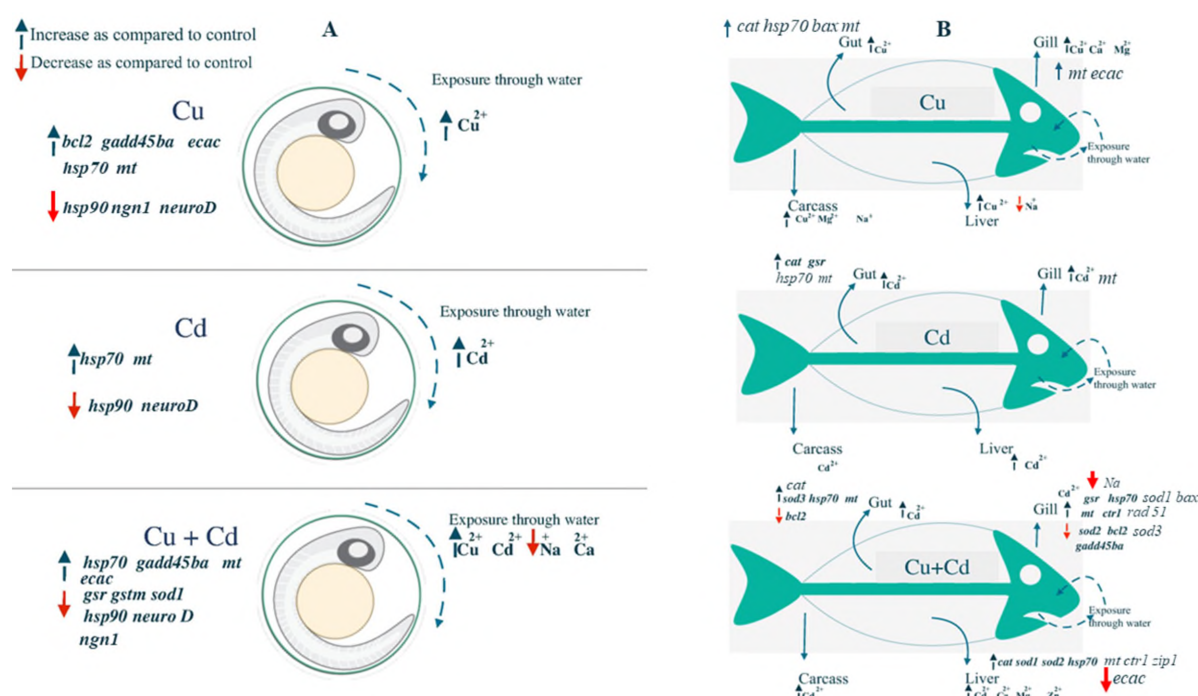

Fig. 7. Diagrammatic presentation of metal accumulation and gene expression as observed in zebrafish (A) embryos (B) gills, gut and liver and carcass of adults.

Conclusion

In the present study, we investigated the effects and underlying mechanisms of single and combined exposure to Cu and Cd in embryos and adult zebrafish. Our results show strong impacts of combined Cu and Cd toxicity on both the development of zebrafish embryos and the functionality of various organs in adult stages, effects that were either less pronounced or absent in single exposures. In embryos, we observed a range of developmental alterations associated with Cu-induced oxidative stress, which was further exacerbated in the presence

of Cd. This interactive toxicity was consistent with behavioral changes in co-exposed embryos, suggestive of potential neurotoxic effects. In adult zebrafish, we identified the gills and gut as particularly sensitive to the toxicity of Cu and Cd. Oxidative stress emerged as a central mechanism underlying toxicity in both embryonic and adult stages, but different defence strategies were activated. A consistent upregulation of other stress response genes, such as hsp70 and mt, was observed in both embryos and adults, Our results challenge the simplistic notion that metal accumulation alone serves as a reliable predictor of toxicity, emphasizing the critical importance of considering internal dynamics. It is important to acknowledge some limitations of our study. Firstly, the exposure concentration used in the mixture scenario was based on the doses used in single exposures, which may not accurately represent equitoxic doses in the combined scenario. Future studies should explore a broader range of concentrations, including those lower than the ones used in single exposures, to gain a more comprehensive understanding of mixture toxicity. Additionally, while we have focused on the combination of Cu and Cd in zebrafish, further research on the toxicity of other metal combinations and their effects on different species is warranted. This will help to fully elucidate the complexity of mixture toxicity in the aquatic environment and provide a more robust basis for toxicological risk assessments.

Supplementary Data

Supplementary Table S1. MIQE guidelines concerning qPCR experiment.	
Experimental design	
Definition of experimental and control groups	Experimental groups - Zebrafish embryos: 96 hour exposure to 0.85 µM Cu, 0.25 µM Cd singly and as a co-exposure. - Zebrafish adults: 7 day exposure to 0.85 µM Cu, 0.25 µM Cd singly and as a co-exposure. - Control groups: zebrafish embryos in culture medium, zebrafish adults in plain medium.
Number within each group	$n \geq 6$
Sample	
Procedure and / or instrumentation	Frozen animals were disrupted by chemical lysis in 200µl RNA lysis/binding buffer (Qiagen, catalogue number 79216). RNA was isolated using a phenol-chloroform extraction procedure and was precipitated with Na-acetate and 70% ethanol and resuspended in RNase free water.
Details of DNase or RNase treatment	Genomic DNA was removed with the Turbo DNA free kit (Ambion® Thermo Fisher Scientific).
Nucleic acid quantification	Nucleotide concentrations were assessed on the Nanodrop ND-1000 spectrophotometer (NanoDrop® ND-1000, ISOGEN Life Science).
Purity	260/280 and 260/230 analysis
Reverse transcription	
Complete reaction conditions	cDNA was synthesized using Superscript TM III first-strand synthesis supermix (Thermo-fisher Scientific)
Amount of RNA and reaction	200ng of total RNA in a reaction volume of 20 µl
Storage condition of cDNA	cDNA was diluted to 1:9 in molecular water before storage at -20 °C
qPCR protocol	
Complete reaction conditions	SYBR Green Master Mix (Applied Biosystems)
Reaction volume and amount of cDNA	Reaction volume: 10 µl cDNA: 2.5 µl
Primer	0.3mM of forward and reverse primer
Polymerase, Mg2+. dNTP buffer	Included in SYBR Green Master Mix (Applied Biosystems, Thermo Fisher Scientific, US)
Complete thermocycling parameters	Universal cycling conditions: 10 minutes at 95⁰ C 40 cycles: 15s at 95⁰ C and 60s at 60⁰ C
Manufacturer of qPCR instrument	ABI PRISM 7500 (Applied Biosystems)
qPCR validation	
Specificity (gel, sequence, melt or digest)	Samples with a melt temperature T_m deviating from the product specific T_m were excluded.
For SYBR Green I, Cq of the NTC	NTC's gave no amplification or at least 5 cycles higher than the highest sample Ct value
PCR efficiency	0.85 1.15
R^2 of calibration curve	≥ 97%
Data analysis	
qPCR analysis program (source)	qBase (Biogazelle)
Method of Cq determination	Hellemans et al., 2007
Results for NTCs	NTC's gave no amplification or at least 5 cycles higher than the highest sample Ct value
Justification of number and choice of reference genes	geNorm analysis
Statistical method for results	Non-parametric
Software (source, version)	Prism GraphPad 8.1.2 (GraphPad software, CA, US).

Table S2. Metal accumulation (µg gfw^{-1}) in zebrafish embryos in different experimental groups at 96 hours post fertilisation (hpf) period. The values are the average ± standard error of mean (SEM) of minimum ten biological replicates in each exposure group (pooled data of two independent experiments. All the metal present in the experimental media were in the dissolved phase and the total measured metal concentration was 94 – 100% of the desired nominal concentrations. A non-parametric test (Kruskal-Wallis one way ANOVA) based on ranks was performed and Dunn's method was used for multiple comparison. *$p<0.05$, **$p<0.01$. fw = fresh weight of the tissue

Metals	Control	0.80 µM Cu	0.25 µM Cd	Cu+Cd
Cu	0.18 ± 0.03	5.43 ± 0.83**	0.85 ± 0.16	5.93 ± 0.36*
Cd	0.04 ± 0.01	0.04 ± 0.01	0.19 ± 0.03**	0.15 ± 0.03*
Na	124.4 ± 14.07	92.40 ± 17.07	132.5 ± 23.67	67.44 ± 5.39*
Mg	46.98 ± 6.97	42.89 ± 8.45	52.00 ± 8.84	36.75 ± 2.52
K	493.6 ± 65.59	378.8 ± 74.63	503.5 ± 67.17	327.3 ± 20.66
Ca	68.48 ± 12.62	32.41 ± 5.11	39.29 ± 8.99	13.38 ± 3.09**
Fe	3.97 ± 1.2	2.76 ± 0.5	2.94 ± 0.2	1.97 ± 0.1
Zn	8.91 ± 2.7	7.32 ± 1.9	6.27 ± 0.5	4.33 ± 0.2

Table S3. Accumulation of metals (µg gdw^{-1}) in gills, liver, gut, and carcasses of adult zebrafish in three experimental groups after 7 days exposure. The values indicated in the table are the average ± standard error of mean (SEM) of ten independent biological replicates. A parametric test was used to compare the treatment and control groups. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ***$p<0.001$ "a" "b" and "c" represent significant values as compared to 0.80 µM Cu, 0.25 µM Cd and the co-exposure respectively.
dw = dry weight of the tissue.

Metals in Gill	Control	0.80 µM Cu	0.25 µM Cd	Cu+Cd
Cu	7.32 ± 0.64	20.76 ± 5.41*$^{b*\ c*}$	8.26 ± 2.06	7.57 ± 0.96
Cd	0.23 ± 0.03	0.13 ± 0.03	5.08 ± 0.57***$^{a***\ c****}$	5.86 ± 1.28 ****
Na	5929 ± 1082	7337 ± 1059	4191 ± 570	3791 ± 516*^{a*}
Mg	1532 ± 327	2839 ± 300*^{b*}	1469 ± 235	1955 ± 267
K	8853 ± 1599	10079 ± 1496	7324 ± 881	7470 ± 864
Ca	60899 ± 16742	128017 ± 18440*^{b**}	45980 ± 11184	63982 ± 9278^{a*}
Fe	242.4 ± 64	159.9 ± 31	164.3 ± 19	142 ± 17
Zn	188.2 ± 43	273 ± 35	184.2 ± 26	185.3 ± 32
Metals in Liver	**Control**	**0.80 µM Cu**	**0.25 µM Cd**	**Cu+Cd**
Cu	4.15 ± 0.28	44.09 ± 9.05****$^{b**\ c**}$	19.45 ± 3.4	20.31 ± 3.4
Cd	0.27 ± 0.05	0.32 ± 0.03	0.99 ± 0.12 ***^{a***}	1.03 ± 0.16****^{a**}
Na	1754 ± 484	781.6 ± 150*	839.2 ± 108	1215 ± 163
Mg	273 ± 65	206.2 ± 45	257.6 ± 43	548.4 ± 70**$^{a***\ b*}$
K	2789 ± 709	1661 ± 466	2584 ± 466	4663 ± 740^{a***}
Ca	304.9 ± 51	1297 ± 493	682 ± 210	3133 ± 1441*^{b*}
Fe	118.5± 19	122.5 ± 11	109.4 ± 17	124.9 ± 13
Zn	37.55 ± 5.6	31.53 ± 4.4	30.32 ± 4.4	78.05 ± 15**$^{a**\ b**}$
Metals in Gut	**Control**	**0.80 µM Cu**	**0.25 µM Cd**	**Cu+Cd**
Cu	38.92 ± 4.0	445.5 ± 91****$^{b****\ c***}$	47.01 ± 3.2	149.3 ± 30
Cd	2.92 ± 0.72	2.08 ± 0.37	95.72 ± 16.5****^{a***}	68.87 ± 13***^{a****}
Na	4932 ± 436	4959 ± 667	5528 ± 315	3253 ± 421^{b*}
Mg	1143 ± 83	1185 ± 178	1397 ± 115	1167 ± 197
K	13283 ± 994	12317 ± 1888	15483 ± 1551	9213 ± 1484^{b*}
Ca	8745 ± 5593	4933 ± 1370	3963 ± 1395	6180 ± 3275
Fe	283.7 ± 42	400.2 ± 79	327.3 ± 38	281.3 ± 47
Zn	150 ± 14	164.5 ± 30	179.8 ± 10	139.2 ± 22
Metals in Carcass	**Control**	**0.80 µM Cu**	**0.25 µM Cd**	**Cu+Cd**
Cu	1.26 ± 0.09	3.07 ± 0.57***$^{b***\ c**}$	1.13 ± 0.06	1.55± 0.09
Cd	0.05 ± 0.004	0.06 ± 0.009	0.60 ± 0.10****$^{a****\ c**}$	0.32 ± 0.04**^{b**}
Na	970.6 ± 36	1462 ± 185*$^{b*\ c**}$	1002 ± 51	872.5 ± 36
Mg	468 ± 10	657.1 ± 18****^{b***}	513 ± 18^{a***}	511 ± 14^{a***}
K	3293 ± 117	3506 ± 144	3219 ± 91	2923 ± 114^{a**}
Ca	16022 ± 2057	22952 ± 3386	15597 ± 1026	16316 ± 484
Fe	22.4 ± 0.5	30.6 ± 4.1	23.73 ± 1.7	19.42 ± 0.8^{a*}
Zn	86.38 ± 9.3	137.1 ± 22$^{b*\ c*}$	88.7 ± 5.8	86.91 ± 3.9

Chapter 2

Water-flea *(Daphnia magna)*

Chapter 2. Exposure pathways influence the toxicity of Cu and Cd: A study on *Daphnia magna*

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Abstract

The toxicity of chemical mixtures is currently the subject of extensive research. However there is less documentation and understanding of age-specific sensitivities to metal mixtures, the importance of different exposure pathways and the molecular events underlying the metal mixture toxicity. In this study, the age-specific toxicity of a copper (Cu) and cadmium (Cd) mixture in *Daphnia magna* across different exposure pathways were compared. The purpose of separating the exposure pathways, was to evaluate the relative importance of each pathway for toxicity. Adult *D. magna* (20 – 21 days old) and neonates (less than 24 hours old) were exposed to Cu and Cd individually and as a binary mixture for 7 days, through three pathways: aqueous (dissolved in water), dietary (spiked with food) and their combination (aqueous + dietary). Our results show that simultaneous exposure to Cu and Cd increased the stress level in both age groups of *D. magna* compared to the single metal exposures. However, neonates showed lower stress tolerance when exposed to similar metal concentrations than adults. The severity of stress varied between exposure pathways and was higher when metal treatments in water and diet were combined. A disturbed redox balance was observed after Cu and Cd co-exposure in both neonates and adults. The results emphasis the vulnerability of organisms to toxic metal mixtures and delineate the importance of exposure pathways in the toxicity assessment of metals in freshwater systems.

1. Introduction

The toxicity of metals and other chemical mixtures remain a concern for scientists and regulators. The release of metals into the environment threatens the well-being of various life forms and ecosystems (Tchounwou et al., 2012; Masindi and Muedi, 2018; Briffa et al., 2020). Previous research has shown that the toxicological profiles of metal mixtures differ from those of single exposures (Barbee et al. 2014; Lari et al., 2017; Crémazy et al. 2018; Moyson et al., 2019; Pilehvar et al., 2019, 2020; David and Cosio, 2021; Majid et al., 2022). However, the effects of metal mixtures across different exposure pathways or life stages of organisms remains less explored. In aquatic systems, metals are either dissolved in water or associated with organic matter or particles, and can be taken up via water or as food. As it is generally believed that the accumulation and toxicity of metals in aquatic organisms occurs primarily through aquatic exposure, water quality criteria have been established based on toxicity tests for dissolved metals. However, a considerable amount of literature has now been published on the toxicity of metals associated with their dietary intake (DeForest and Meyer, 2015; Bhuvaneshwari et al., 2018; McDonald, 2021). The effects of metals through combined exposure pathways have been previously studied (Sofyan et al., 2007; Geffard et al., 2009), but not in the context of metal mixtures or at different life stages. While it is generally assumed that early life stages are more sensitive than later life stages (Pineda et al., 2012; Holan et al., 2018; Majid et al., 2022), there are also studies showing similar or greater sensitivity in later life stages than earlier life stages (Mohammad, 2013; Wise, 2022). In this perspective, the current study was conducted to compare age-specific susceptibility to the toxicity of metal mixtures across multiple exposure pathways. The present study compares copper (Cu) and cadmium (Cd) as toxicants in the freshwater microcrustacean, *Daphnia magna*. *D. magna* is a recommended test organism in standard OECD testing protocols (OECD, Test No. 202; OECD Test No. 211) and is widely used in ecotoxicological studies due to its metal sensitivity, short life cycle, and ease of cultivation in the laboratory (Vandenbrouck et al., 2010; Kim et al., 2015; Tkaczyk et al, 2021). Moreover, *D. magna* shares many genes with higher organisms and is an important model organism in environmental genomics to better understand the effects of pollutants on cellular and molecular processes (Lee et al., 2019).

In this study, Cu and Cd were selected as toxicants based on their frequent occurrence in freshwater systems, persistence in different environmental compartments, distinct chemical properties, mode of action, and ecological impact (ATSDR, 2004, 2012; Sadeq and Beckerman, 2020; Arambawatta-Lekamge et al., 2021). Copper is a redox–active metal, essential for organisms, and involved in several biological processes in all living organisms (De Romaña et al., 2011; Osredkar, 2014; Kardos et al., 2018; Fan et al., 2020; Ruiz et al., 2021). However, at elevated concentrations, it can become a toxicant and pose health risk to both humans and aquatic organisms (Fan et al., 2020; Royer and Sharman, 2023;). The toxicity of Cu has been intensively studied on several aquatic species and there is evidence showing higher risk of Cu to aquatic species than other priority metals such as Cd, Hg and Pb (Fu et al., 2016; Liao et al., 2023). Several mechanisms have been proposed to describe Cu-induced toxicity in aquatic organisms. These include disruption of sodium balance through inhibition of the Na^+/K^+-ATPase enzyme, effects on bioenergetics; and oxidative stress that triggers cell damage and death (Brix et al., 2022; Liao et al., 2023). Cadmium is biologically non-essential, and a priority pollutant known to pose a risk to human health and aquatic organisms (Liu et al., 2022). The toxicity of Cd is comparatively lower than Cu, but it has longer biological half-life and low excretion rate, as a result it may occur at elevated concentrations in aquatic environments, leading to its accumulation in food chains (Mandich, 2018; Liu et al., 2022; Lee et al., 2023). Due to the accumulation capacity of Cd in animal tissues, it can exhibit high toxicity even at low levels (Mashhadikhan et al., 2022; Lee et al., 2023). Cadmium is known to disrupt the cellular homeostasis of essential elements (such as calcium (Ca), iron (Fe) and zinc (Zn) and indirectly cause oxidative stress by disrupting the cellular redox balance in favour of pro-oxidants (Martelli et al., 2006; Cuypers et al., 2010; Branca et al., 2020; Lee et al., 2023).

In the present study, we aimed to investigate the combined effect of Cu and Cd and exposure pathways in *D. magna* adults and neonates, representing two age groups. The study was conducted by dividing it into three separate exposure setups with aquatic (metals dissolved in water), dietary (metals spiked with food) and a combination of both (aqueous + dietary) exposures to assess the toxicity response after single and co-exposure to Cu and Cd. The toxicity indicators including growth, reproduction and survival were used to evaluate the individual and combined effects of metals and exposure pathways. This was accompanied by measuring the metal accumulation in both adults and neonates. The analysis was completed by the determination of the transcription level of genes encoding for proteins

involved, antioxidant activity, apoptosis, DNA repair mechanisms, reproduction, development, and metal transport. Finally, the cellular redox state of animals was studies.

2. Material and methods

2.1 Experimental design

The aim of the experiments was to investigate the response of single and co-exposure to Cu and Cd under different exposure setups in adults and neonates of *D magna*. In the first exposure setup, adults and neonates were exposed to Cu and Cd separately and as a mixture through water (aqueous exposure) (concentration addition exposure). Exposure concentrations were determined by the OECD Test Guidelines No. 202 (OECD, 2004) (see section 2.3). In the second exposure setup the animals were exposed to the metals through food (dietary exposure) (concentration addition exposure). In this experiment, Cu- and Cd-spiked green algae *Raphidocelis subcapitata* were utilized as food. To determine how much metal was taken up by the algae, experiments on metal uptake were carried out first with *R. subcapitata* (see section 2.4) followed by exposure of animals with the metal loaded food. The two exposure pathways were combined in the third experimental setup to assess the influence of the two pathways on toxicity. All the experiments were conducted over a period of 7 days and toxicity indicators such as growth, reproduction and survival were monitored. Genes encoding proteins involved in oxidative stress, apoptosis regulation, DNA repair, development, and metal transport were studied and the redox state was evaluated. The overview of the experimental setup is presented in figure 1.

2.2 Daphnia magna culture

The animals used in the study originated from a healthy *Daphnia magna* stock reared in our laboratory (originally supplied by University of Ghent, Belgium). The stock culture was maintained under controlled laboratory conditions in medium hard reconstituted water (80 – 100 mg/L as $CaCO_3$) in one liter polypropylene aquaria at a constant temperature (20 ^{0}C ± 1 ^{0}C), pH (8.0 ± 0.2) and a 14h:10h, light: dark photoperiod. Daphnids (less than 24 hours old) were acclimatised for at least three weeks (one generations) prior to the start of the experiments to avoid stressing them. Given our aim to compare toxicity responses between early developmental and adult life stages, fully mature females (20 to 21 days old), that have completed several molting cycles and newly hatched offspring (neonates) which were less than 24 hours old were specifically used in the study. The offspring (neonates) produced during rearing period were discarded daily, except the last brood (6th brood), which was

used in the study. The water was refreshed three times a week by transferring daphnids to new medium using pipettes (internal diameter at least 1.5 times the size of the animals). Care was taken to ensure that the animals are not bumped or crushed during transfer to the new medium. The animals were introduced below the surface of the new medium to avoid trapping air under their carapace. During rearing period, the daphnids were fed a mixture of the algae *R. subcapitata* and *Chlamydomonas reinhardtii* at a ratio of 3:1 (2 x 10^7 algal cells / daphnid.

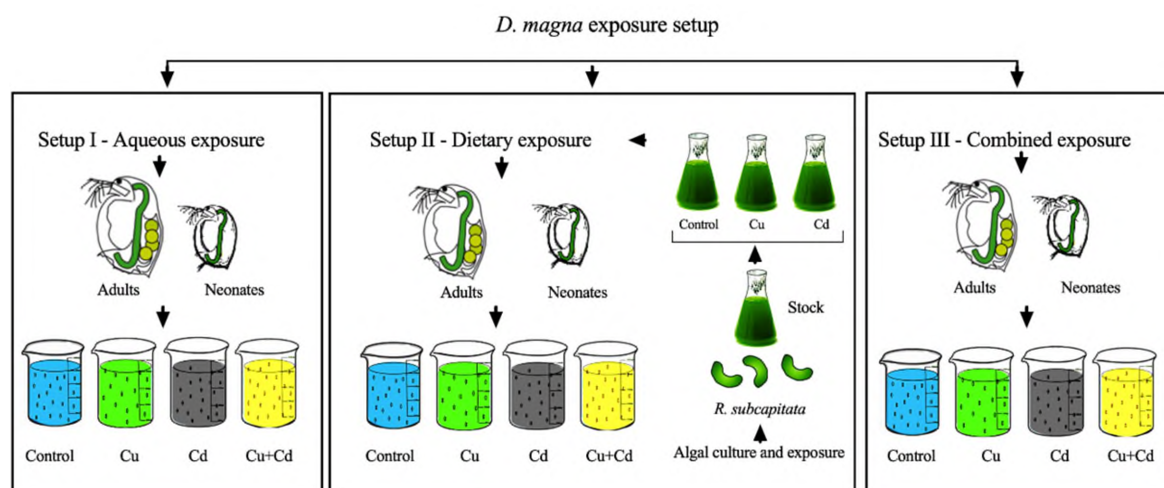


Fig. 1. Overview of experimental design. *D. magna* adults (20-21 days old) and neonates (less than 24 h old) were exposed to the single and binary mixture of Cu and Cd via three exposure set-ups: aqueous (dissolved in water), dietary (spiked with food) and their combination (aqueous + dietary), over 7 days.

2.3 Selection of exposure concentration

The exposure concentrations were selected based on the OECD acute immobilization test (OECD, 2004). The acute toxicity of the individual metals and the metal mixtures present in the dissolved phase was determined in a 48-hour static, non-renewal lethality test in accordance with the test guidelines (Test No. 202, OECD, 2004). One day prior to the start of the toxicity test, a one-molar stock solutions (1M) of $CuSO_4$, and $CdCl_2$ was prepared in the same water that was used for culturing *D. magna* and diluted to concentration ranges of 0.15 – 3.0 µM $CuSO_4$, and 0.02 – 1.0 µM $CdCl_2$. These ranges were selected based on the published data on Cu and Cd toxicity to *D. magna* (Canizares-Villanueva et al., 2000; Haap and Kohler, 2009; Komjarova and Blust, 2009; Kim et al., 2017; Lari et al., 2017). The test ranges (Cu = 0.15, 0.25, 0.35, 0.50, 0,75, 1.0, 1.5, 2.0, 2,5 and 3.0 µM; Cd = 0.02, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.5, 0.75, and 1 µM) were applied in a gradient to produce 0% to 100% mortality. A total of 20 organisms (24 hours old neonates) were exposed to each metal

concentration (singly and in combination) and a control solution. Each metal concentration was tested in 4 replicates in 50 ml polycarbonate cups containing 25 ml of test solution (5 neonates/replicate). After 48 hours, the immobilised neonates were counted as an indicator of mortality. Animals were considered immobilised if no movement was observed for 15 seconds even after gentle probing. The test was repeated twice. Lethal concentrations were determined by plotting the dose – response curve using Prism8 software (GraphPad)was calculated with 95% confidence interval. Exposure concentration for single and mixture treatments were selected based on 20% of 48h LC_{50} (Cu = 0.25 µM, Cd = 0.02 µM).

2.4 Metal uptake experiments with *R. subcapitata*

Metal uptake experiments were conducted with green algae *R subcapitata* to determine the concentration of metals absorbed by algae. The algae were cultured according to the OECD Test Guidelines 201 (OECD, 2011). The log-phase algae were exposed to 0.15 µM $CuSO_4.5H_2O$ [96 h IC_{50} = 1.38 µM (De Oliveira-Filho et al., 2004)] and 0.05 µM $CdCl_2$. H_2O [96 h IC_{50} = 0.67 (Reis et al., 2021)] with two replicates per condition in glass flasks of one litre capacity. Exposures were continued for 7 days before they were fed to animals. This is also the time required for completion of 7 days algal growth cycle. The experiments were conducted under continuous illumination and aeration at 20 ± 1^0C. To avoid contamination, the opening of each flask was sealed with aluminium foil and air was filtered through a 0.22 µm nylon filter for aeration. The number of algal cells was monitored daily in each test solution using a Beckman Multisizer Z3 Coulter Counter (Beckman Coulter, Inc. Version 3.33). The metal content of each replicate was determined each day according to the procedure followed by Komjarova and Blust (2009b). Briefly, 12 mL of the culture was withdrawn from each stock. Ten ml of culture was withdrawn to determine the number of algal cells using a Beckman Multisizer Z3 Coulter Counter (Beckman Coulter, Inc. Version 3.33). The remaining 2 mL of culture was taken in a separate 50 ml tube, raised to the 40 ml mark with high purity (Milli-Q®) water and used for metal analysis of algae. This was followed by shaking and centrifugation. After centrifugation, the supernatant was discarded, and the pellet was oven-dried at 60 °C for 24 hours. After drying, the samples were digested in 200 µL of ultra-pure nitric acid (Trace metal grade - Merck, Darmstadt, Germany) on a hot block at 110 ^{0}C for one hour. Digested samples were diluted to 40 mL with ultrapure water (Milli-Q, Bedford, MA, USA). The Cu and Cd concentrations of the samples and blanks were analysed by inductively coupled plasma mass spectrometry (7700x ICP-MS, Agilent Technologies). The exposure resulted in a Cu load of 2.2×10^{-4} ng/cell dry weight) and a Cd

load of 2.9 x 10^{-6} ng/cell dry weight). A reference standard mussel tissue (NIST - 2976) was used to assess the validity of the metal analysis protocol.

2.5 Daphnia magna exposure

For the aqueous exposure, four exposure conditions were chosen: a control (without added metals), 0.25 µM Cu, 0.02 µM Cd singly and a mixture (i.e. concentration addition exposure) Both control and metal-added solutions were prepared from 1M stock solutions, one day before the start of exposure. For each exposure category, 100 adult animals were separately transferred to one liter glass jars filled with 800 ml of exposure medium. To prevent binding of metals to feed particles, food was not added to the aqueous media during the uptake period. The medium was renewed three times per week. For dietary exposure, metal-spiked algae *R. subcapitata* containing 2.2 x 10^{-4} ng Cu/cell dry weight (equivalent to 4.33 x 10^{3} ng Cu/daphnid) and 2.9 x 10^{-6} ng Cd/cell dry weight (equivalent to 5.72 x 10^{1} ng Cd/daphnid) were fed daily to the respective exposure groups while, control animals were fed uncontaminated diet (without added metals). In the combined scenario the animals were simultaneously exposed to water containing metal concentrations used for aqueous exposure, as well as a diet containing concentrations used in dietary exposure individually (refer to aqueous and dietary exposure details above). In all exposure groups, survival rate and number of neonates produced per female daphnid were monitored daily and collected from the medium. Water quality variables (pH, temperature, dissolved oxygen, and conductivity) and mortality were recorded daily. Animals were considered dead if no movement was observed for 15 seconds even after gentle shaking. To determine background metal concentrations, 10 ml water samples were daily collected from each exposure medium. Samples were filtered through a 0.22 µm filter, acidified with 2% HNO_3 and quantified using inductively coupled plasma mass spectrometry (7700x ICP-MS, Agilent Technologies). An additional 10 ml of water was taken before and after each feeding to determine the number of algal cells consumed, using a Beckman Multisizer Z3 Coulter Counter (Beckman Coulter, Inc. Version 3.33).

2.6 Metal accumulation analysis

All animal samples were analysed for metal accumulation using inductively coupled plasma mass spectrometry (7700x ICP-MS, Agilent Technologies). At the end of the exposure period, animals were collected (8 samples x 10/replicate) in 1.5 ml Eppendorf tubes and rinsed with deionised water. After the samples were rinsed, the remaining water was removed using a pipette and then snap frozen in liquid nitrogen. The fresh weight of all samples was then

measured. The tissues were thawed, and oven dried at 60 °C for 24 hours. After drying, all tissues were digested in 150 µL ultrapure nitric acid (trace metal grade - Merck, Darmstadt, Germany) by microwave-assisted digestion at 100 W (3x3 min), 180 W (3x3 min) and 300 W (1min). Digestates were diluted up to 4 mL with ultrapure water (Milli-Q, Bedford, MA, USA). The Cu and Cd concentrations of the animal samples and 3 blanks were analysed. Concentration was expressed as µg per gram fresh weight (µg gfw^{-1}) (dry weight: fresh weight = 0.01: 0.33). A reference standard mussel tissue (NIST - 2976) was used to assess the validity of the protocol.

2.7 Growth, reproduction, and survival analysis

Growth was determined at the end of exposure period by measuring the length of the carapax from the top of the head to the base of the daphnid spine (10/condition). The measurements were done by capturing digital images using a CCD camera (DFK 41AF02 FC, Imaging source) mounted on a trinocular stereomicroscope (Nikon, SMZ 800). The pictures were processed to measure length using ImageJ software (version 1.52u, National Institutes of Health, Bethesda, MD, USA). For the assessment of reproduction, adult daphnids were kept in each exposure condition individually (10/condition) and the number of live offspring per female daphnid were counted daily and then removed. During the entire exposure period, mortality was determined by counting the number of dead animals on each day. Animals were considered dead when no movement occurred even after gentle shaking.

2.8 Molecular and cellular analysis

2.8.1 Gene expression

Gene expression was evaluated in adults and neonates in all the exposure setups. The nucleotide sequences of some of the selected genes were obtained from NCBI database (https://www.ncbi.nlm.nih.gov/) (Table S1: see supplementary data). Other primer sequences used were obtained from previously published data (Heckmann et al., 2006; Vandenbrouck et al., 2009; David et al., 2011; Kim et al., 2017, 2018; Liu et al., 2019). To optimize the design of the primers across exon boundaries, primers were first manually designed for each gene and then subsequently analysed using the primer analysis software LightCycler® probe design software (version 3.5, Roche Molecular Biochemicals, Germany). Primer efficiencies between 85 – 100% as determined by using the Ct slope method. All primer sequences are listed in Table 1.

RNA was extracted using a phenol-chloroform extraction procedure (Chomczynski and Sacchi, 2006) and precipitated using Na-acetate and 70% ethanol. RNA concentration and quality were determined spectrophotometrically using Nanodrop ND-1000 (NanoDrop® ND-1000, ISOGEN Life Science). All RNA samples were adjusted to a concentration of 200 ng. Genomic DNA was removed using Turbo DNA free kit (Ambion® Thermo Fisher Scientific). After RNA extraction, cDNA synthesis was performed using the Super-script TM III first-strand synthesis supermix (Thermo-fisher Scientific) according to the manufacturer's instructions. cDNA samples were stored at -20 °C until their use for amplification by the real-time PCR.

Real-time qPCR was performed in a 96-well plate (Applied Biosystems, Thermo Fisher Scientific) using the Fast Syber Green master mix (Applied Biosystems, Thermo Fisher Scientific) amplified and detected by using the 7500 Fast Real-time PCR system (Applied Biosystems, Life Technologies). Primer efficiencies calculated as $E=10^{-1/slope}$, were evaluated using four-point standard curves, prepared by a 1:3 serial dilution of cDNA. Efficiencies ranging from 0.85 to 1.15 were accepted. Potential reference genes were selected according to the method given by Rongying et al (2007) (see Table 1). GeNorm was used for the analysis of the most stable reference genes. Gene expression analysis was performed following MIQE guidelines (Bustin et al., 2009) (Table S2: see supplementary data). Gene expression (transcriptional) profiles of the genes related to the cellular redox state, apoptosis, DNA repair mechanisms, reproduction, development and metal transport were studied in all exposure setups. The relative quantification of each gene expression level was normalized according to the expression of the at least three most stable reference genes.

2.8.2 Hydrogen peroxide quantification

The concentration of hydrogen peroxide (H_2O_2) (8/condition) was determined spectrophotometrically using an OxiSelect™ hydrogen peroxide assay kit (Cell Biolabs, Inc., San Diego, US) following the instructions provided by the manufacturer. Briefly, daphnids (3-4 animals/sample) were snap-frozen in liquid nitrogen and homogenised (using glass beads) in a 1x buffer. The homogenates were centrifuged at 13,000 revolutions per minute (rpm) for 1–2 min at 4 °C and the supernatant obtained was used for the quantification of H_2O_2. A small volume of the supernatant (10 µL) was diluted in a ratio of 1:10 and used for protein estimation. Next, ADHP (10-Acetyl-3, 7-dihydroxyphenoxazine) and HRP (horseradish peroxidase) working solutions were prepared. The ADHP/HRP working solution

was added (50 µL) to each sample and incubated at room temperature for 30 minutes in dark. The absorbance was measured at 544 nm in a FLUOstar Omega multi–mode microplate reader (BMG Labtech, Ortenberg, Germany). The H_2O_2 content was determined using a standard curve. The total protein content was quantified separately using Bradford 1X dye reagent and with Bovine Serum Albumin (BSA) serving as the standard.

Table 1. Nucleotide sequence of specific primer pair of *D. magna* used in the study.

Gene	Forward primer	Reverse primer
*Act**	CCACACTGTCCCCATTTATGA A	CGC GAC CAGCCAAAT CC
*cyp**	GACTTTCCACCAGTGCCATT	AAC TTTCCATCGCATCATCC
*18s**	CGCTCTGAATCAAGGGTGTT	TGTCCGACCGTGAAGAGAGT
*28s**	GAGGCGCAATGAAAGTGAAG	TGTTCGAGACGGGATCA
*β-act**	CCACACTGTCCCCATTTATGA A	CGCGACCAGCCAAATCC
*sdh**	TGCCAT TTAGTCGCACTC AG	GTGAGCTTGTCCTCCTTTGC
cat	CCGTTACAACACTGCCGATGA	AAGGCTGTGCGTTCTTTAGATG
CuZn-sod	GAGACCTGGGCAACATTGTG	GACTCTGGCCCGCTAAGTG
Mn-sod	GATGTTTGGGAGCATGCCTAC	GGACCGACACATCTTTCCAG
gst	GGGAGTCTTTTACCACCGTTTC	TCGCCAGCAGCATACTTGTT
grx	TGAAGCAGCTGACCCATAAG	GCAACTTGGTTTCGTTGAGA
gpx	CGTGGCTACTTACTGAGGGTTT	CGGACGAACGTAACGGATT
hsp70	ACTGATGCCGTGATTACTGTTC	CCTTGTGATGCTGGTGTAGAA
hsp90	CCGAGGAAGAGAAACCAAAG	CGTCGACCGAATACTTCTCC
p73-like	GCCTGGGCATTTGAACTTTA	ATGGAAGTGATCAGCCTTGG
riv1	ATGCGTTAGGCGTCAATACC	TACAAGGTTTGCCCTTGCTT
rad17like	GCGAATCATTCAAACCACTAC	CCATTTCTCTGAGTTCGATCT
aif1	CCTTACATGAGGCCACCACT	ACTGAAACGCCACCATTTTC
ai5	TCTGAATGACGCGAAAGATG	ACAGGTCAAACATGGCATCA
vtg	CTG TTC CTC GCT CTG TCT TG	CCA GAG AAG GAA GCG TTG TAG
opsin	TCCTCGTGCTTGAAGAC	GCGCTTGTTTCGGATAC
vri	CATCCACATCACCAGCATCAC	CGCGACAACGACCAATCT
cp	TCTGCTGGGAAAGTTGAAGATG	CAAATGGACGAGAAGACGATG
notch2	GCCACATCGAACACTGAAT	CATGTTGAGCACTTCACCT
mt-a	TTGCCAAAACAATT GCTCAT	CACCTCCAGTGGC ACAAAT
ctr1	GGGAGTACCTGAGTCGGA	ATGTAATGGGACGCACTG
zip9	CGTTCGTATTGTTCCTCTACAG	CCCAAATTACTCTTGCCAC
Serca	TCGCAAAGAAGTCT TCGACTC	AAACACCAATAC GACGGCAG

*Housekeeping genes (HKG)

2.8.3 Glutathione assay

Glutathione content has been utilized as an indicator of metal toxicity, particularly in the context of copper stress (Stoiber et al., 2007). Glutathione, as a key antioxidant molecule, was selected for measurement to assess the antioxidant defense capacity of the daphnids under all metal exposure conditions. To measure glutathione content, a spectrophotometric method was used, based on the method originally described by Tietz (1969). Each daphnid

(8/condition) was photographed to determine the mean total body size (mm^2) for normalisation. Next the daphnids (3-4 animals/sample) were snap-frozen in liquid nitrogen and stored at −70 °C. Frozen animals were dissolved in 320 µl of 0.2 M HCl, equipped with glass beads measuring 2 µm in diameter and then disrupted using a Retsch Mixer Mill MM 200 (Retsch) for one minute at a frequency of 30 Hz. The samples were kept on ice throughout the procedure. After this step, 280 µl of the homogenate was transferred to a new tube, neutralised with 30 µl of 0.2 M NaH2PO4 followed by adjusting the pH to 5.6 with approximately 230 µl of 0.2 M NaOH.

Next, 340 µL of each sample was transferred to a new tube for (oxidised) glutathione, glutathione disulfide (GSSG) analysis and the remaining 180 µL was used for glutathione reductase (GSH) assay. To quantify total GSH, four separate aliquots of 40 µl of the neutralised extract were added in a 96-well plate (Greiner Bio-One, Kremsmünster, Austria). The reaction mixture, with a total volume of 200 µl comprised 0.1 M NaH_2PO_4 (pH 7.5), 5 mM EDTA, 0.5 mM NADPH, 0.6 mM DTNB. Once again, the reaction was initiated by the adding 0.2 U of GR to all but one sample (no-GR control). To quantify GSSG, samples were first treated with 1 µl of 2 -vinyl pyridine (2-VP) for 30 min at RT to complex GSH. In order to remove surplus 2-VP, the solution that had undergone derivatization was subjected to two rounds of centrifugation. Four aliquots of 80 µl neutralised extract were added to 96-plate wells. The total reaction volume of 200 µl also contained 0.1 M NaH_2PO_4 (pH 7.5), 5 mM EDTA, 0.5 mM NADPH, 0.6 mM DTNB. Again, the reaction was initiated by the addition of 0.2 U of GR to all samples but one (no-GR control). After mixing the reaction mixture on a shaker, absorbance (GR-dependent reduction of DTNB) was monitored for 15 min with the Fluostar Omega multi-mode microplate reader (BMG Labtech, Ortenberg, Germany). The standards were run simultaneously alongside the same plates as duplicate assays. A negative control with milliQ water was used as a blank measurement.

2.9 Statistical analysis

Statistical analysis was performed using Prism8 statistical software (GraphPad). In all experiments, each parameter was evaluated separately with an appropriate control. All the experimental results were analysed by a non-parametric test (Kruskal – Wallis and Dunn's multiple comparison test) based on ranking. A p-value less than 0.05 was considered statistically significant.

3. Results

3.1 Metal accumulation

Accumulation of Cu and Cd was determined after adults and neonates were exposed to Cu and Cd separately and in combination via the aqueous, dietary, and combined exposure pathway (Fig. 2 and Fig.3). In both adults and neonates, all exposure pathways resulted in comparable Cu accumulation with single and co-exposure to Cd ($p < 0.05$). In contrast to Cu, the accumulation of Cd in adult animals was generally higher when exposed to Cd alone and decreased in the presence of Cu [aqueous: 25% decrease ($p < 0.05$), dietary: 42% decrease ($p < 0.05$, combined: 37% decrease ($p < 0.05$)]. In neonates, Cd concentrations were significantly increased in both single and mixture-exposed animals ($p < 0.05$). As with the adult animals, neonates generally accumulated less Cd when exposed to the mixture [(dietary: 40% decrease ($p < 0.05$), combined: 11% decrease ($p < 0.05$)], except for the exposure via the aqueous medium, where an increase in Cd concentration was observed (26%, $p < 0.05$). On comparing the metal accumulation between aqueous, dietary and combined pathway, Cu and Cd accumulation from aqueous and combined exposures was higher than from diet only ($p<0.5$) and comparable between aqueous and combined exposure both in adults and neonates. When comparing the metal accumulation between adults and neonates, the accumulation was in general lower in neonates in each exposure condition compared to adults ($p<0.5$).

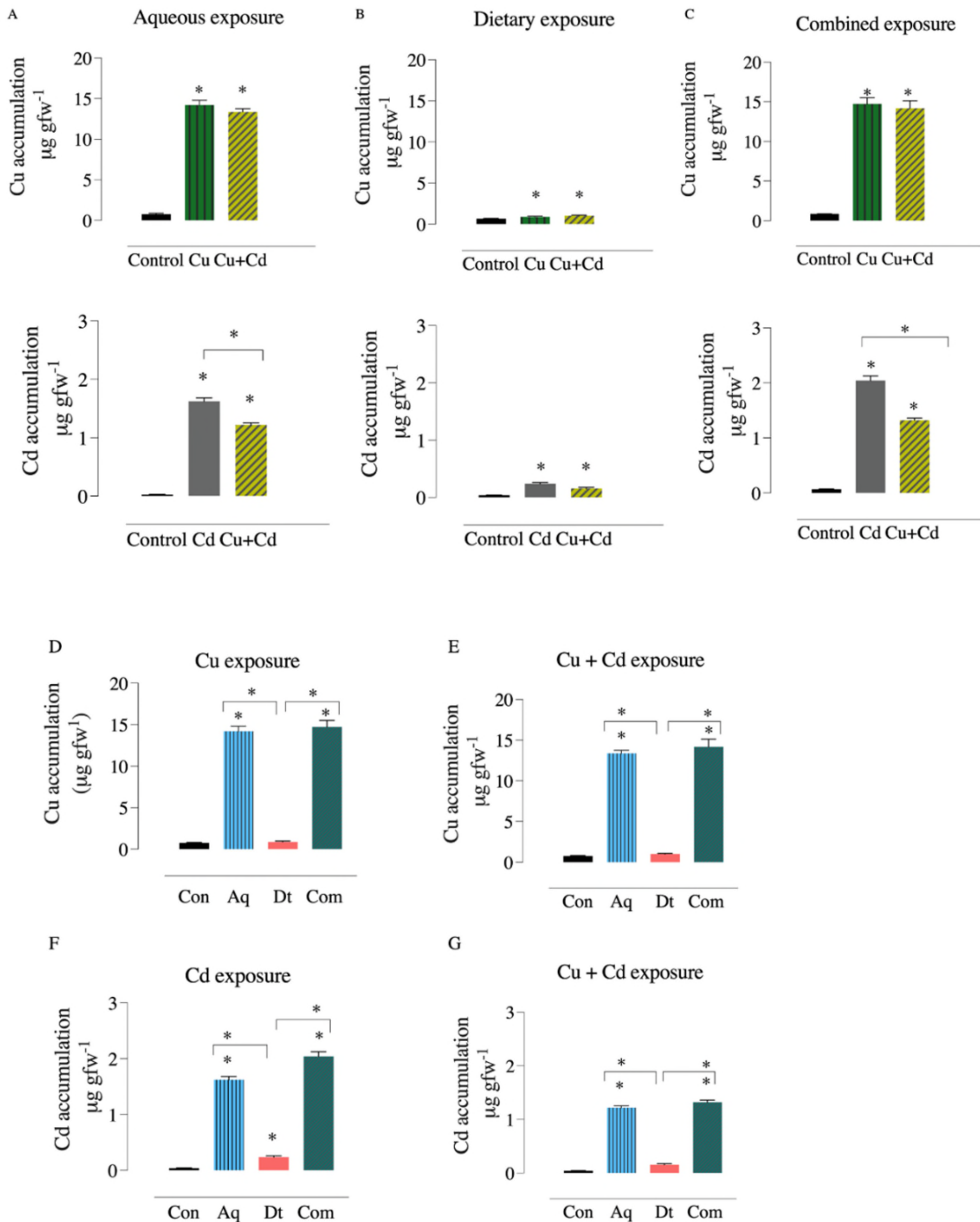


Fig. 2. Metal accumulation in *D. magna* adults after 7 days exposure. Graphs representing metal concentrations in adult animals exposed to Cu and Cd alone and in combination via (A) aqueous, (B) dietary and (C) combined pathway. ((D-G) comparison of metal accumulation among three exposure pathways. The values presented in the figure represent pooled data, showing the average ± standard error of the mean (SEM) from a minimum of 16 biological replicates. Two independent experiments were performed and the results of two experiments confirm each other. A non-parametric test (Kruskal – Wallis and Dunn's multiple comparison test) on ranking was used to compare the treatment and the control groups. fw = fresh weight , *$p<0.05$.*

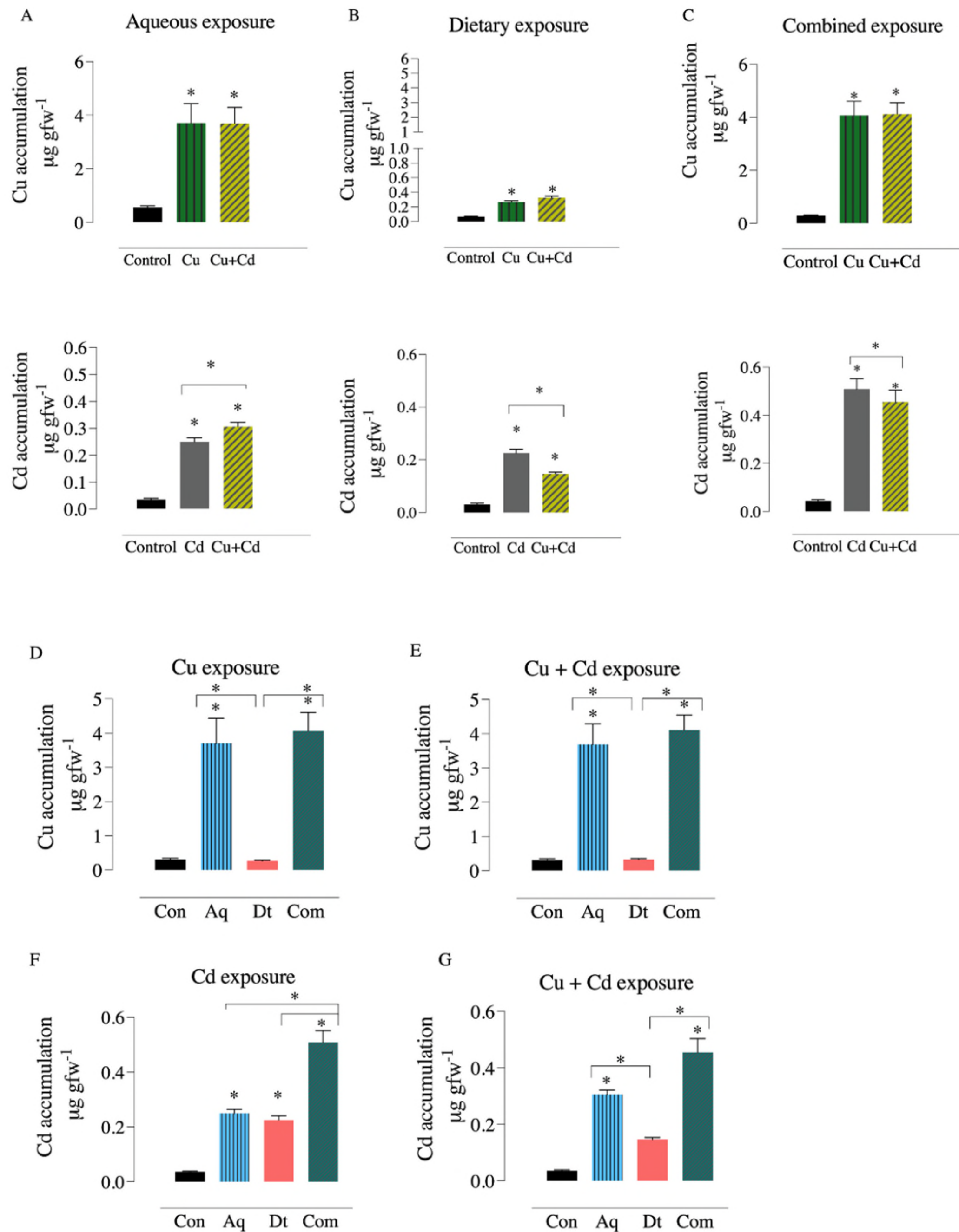


Fig. 3. Metal accumulation in *D. magna* neonates after 7 days exposure. Graphs representing metal concentrations in adult animals exposed to Cu and Cd alone and in combination via (A) aqueous, (B) dietary and (C) combined pathway. (D-G) comparison of metal accumulation among three exposure pathways. The values presented in the figure represent pooled data, showing the average ± standard error of the mean (SEM) from a minimum of 16 biological replicates. Two independent experiments were

performed and the results of two experiments confirm each other. A non-parametric test (Kruskal – Wallis and Dunn's multiple comparison test) on ranking was used to compare the treatment and the control groups. fw = fresh weight , *$p<0.05$.*

3.2 Reproduction, Growth and survival

Reproductive capacity, growth, and mortality were assessed as general toxicity endpoints. Reproduction, measured as the number of neonates produced by each adult female daphnid was a sensitive endpoint and decreased significantly in all metal-exposed animals when exposed via the combined pathway (Cu: $p < 0.05$, Cd: $p < 0.05$, Cu+Cd: $p < 0.001$) (Fig. 4, Table 2). This decrease was higher in animals exposed to the Cu and Cd mixture (52%, $p < 0.001$) than when they were exposed to only one metal (Cu 21%, $p < 0.05$; Cd 17%, $p < 0.05$) at a time. The Cu and Cd mixture also resulted in a significant decrease in reproductive capacity when exposure was via the aqueous and dietary media ($p < 0.05$). On pathway wise comparison, a decrease in reproductive capacity was found in combined exposure irrespective of single or mixed exposure.

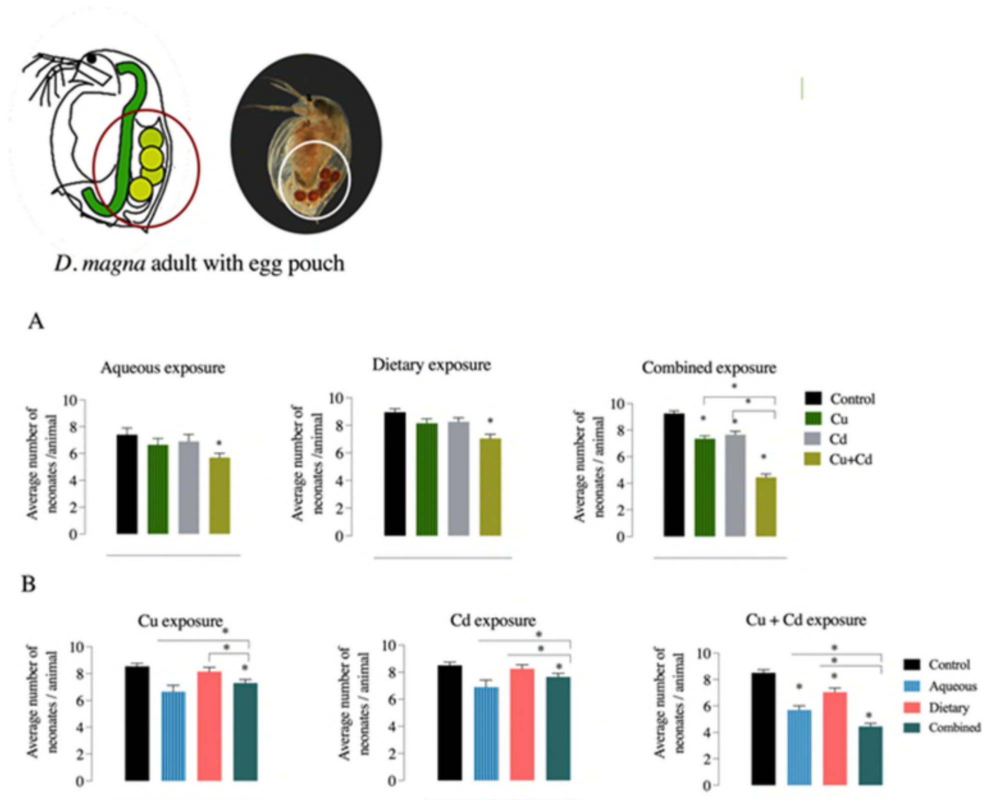


Fig. 4. Reproduction in *D. magna.* The graphs represent; (A) Total reproductive output (number of neonates / animal) in adults exposed to the respective Cu and Cd concentrations alone via aqueous, dietary, and combined media. (B) comparison of reproductive capacity among three exposure pathway. The values indicated in figure

are the average ± standard error of mean (SEM) of minimum 20 biological replicates. Two independent experiments were performed and the results of two experiments confirm each other. A non-parametric test (Kruskal – Wallis and Dunn's multiple comparison test) was used to compare the treatment and the control groups. $*p<0.05$, $**p<0.01$, $***p<0.001$ Scale bar = 500 µm.

In neonates, exposure to the mixture of Cu and Cd impaired growth through all exposure pathways and caused a significant decrease in body size compared to control animals ($p < 0.05$). The decrease in growth was strongest under the combined exposure scenario (aqueous 14%, $p < 0.05$; dietary 9%, $p < 0.05$; combined 30%; $p < 0.05$) (Fig. 5, Table 2). Impairment in growth was also observed in Cu-exposed neonates in aqueous and combined exposure settings ($p < 0.05$).

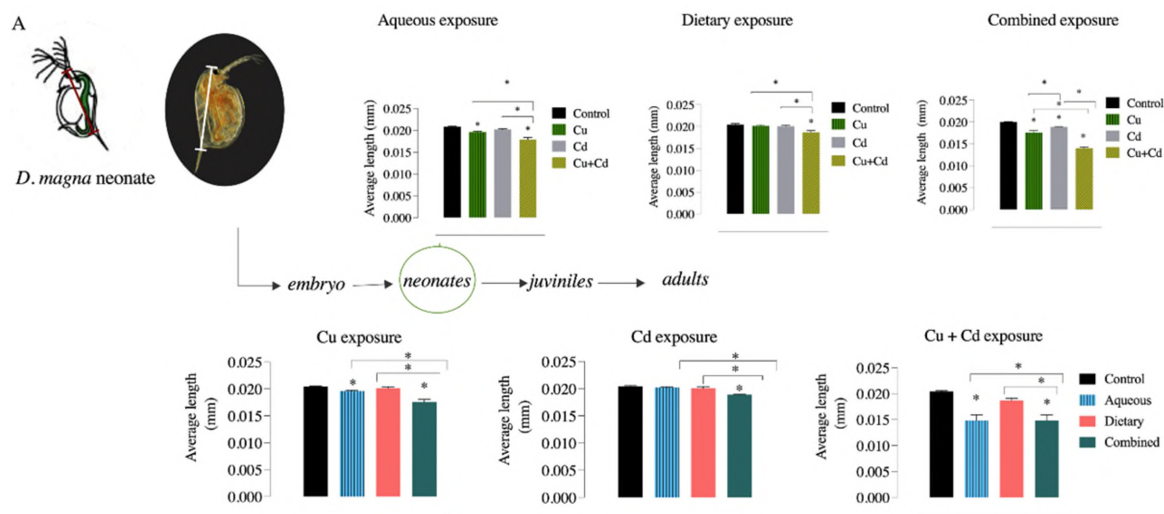


Fig. 5. Growth in *D. magna.* The graphs represent; (A) Growth (length measured in mm) in neonates exposed to the respective Cu and Cd concentrations alone and in a mixture after 7 days exposure via aqueous, dietary, and the combined media. (B) comparison of growth among three exposure pathway. The values indicated in figure are the average ± standard error of mean (SEM) of minimum 20 biological replicates. Two independent experiments were performed and the results of two experiments confirm each other. A non-parametric test (Kruskal – Wallis and Dunn's multiple comparison test) was used to compare the treatment and the control groups. $*p<0.05$, $**p<0.01$, $***p<0.001$ Scale bar = 500 µm.

In adult animals, no mortality occurred throughout the exposure period in any of the exposure groups. In neonates, mortality occurred in all exposure groups when exposure occurred via aqueous and combined. However, the strongest decline in survival was observed in mixture exposed animals via combined medium (30%, $p < 0.05$)

Table 2. Toxicity responses in *D. magna* neonates and adults after single and co-exposure to Cu and Cd across three exposure pathways (aqueous, dietary, and combined). The effect was classified as more than additive when combined effect of the individual exposures was significantly greater (denoted by asterisk sign) than the sum of the effects of individual exposures alone). **p<0.05; ***p<0.001.*

D. magna adults				
Reproduction				
Pathway	Cu	Cd	Cu+Cd	Effect of co -exposure
Aqueous	-10%	-7%	-23%	no significant effect
Dietary	-9%	-8%	-21%	no significant effect
Combined	-21%	-17%	-52%***	more than additive
D. magna neonates				
Growth				
Pathway	Cu	Cd	Cu+Cd	Effect of co-exposure
Aqueous	-6%	-3%	-14% *	more than additive
Dietary	-2%	-2%	-9%*	more than additive
Combined	-12%	-6%	-30%*	more than additive

3.3 Gene expression changes in adults

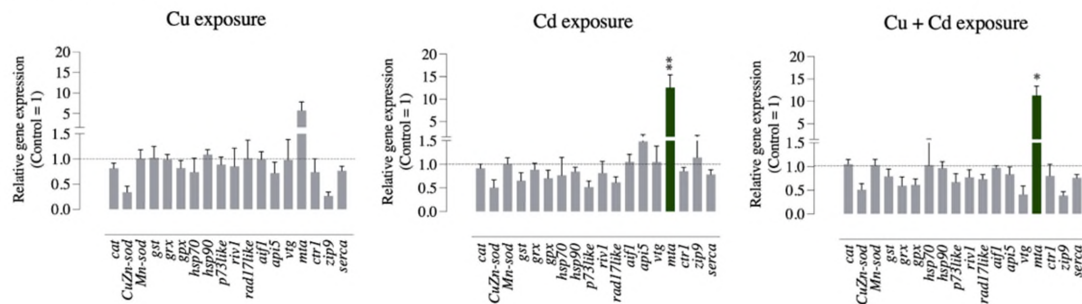
Exposure via the aqueous medium resulted in the induction of metallothionein (*mta*), which was strongly upregulated in Cd ($p < 0.01$) and mixture ($p < 0.05$) exposed animals. In dietary exposed animals, the Cu and Cd mixture caused an upregulation of glutathione transferase (*gst;* $p < 0.05$). In contrast to the other exposure pathways, Cu and Cd mixture exposure via combined exposure pathway caused alterations in the activity of several genes. Genes related to oxidative stress (*Mn-sod;* $p < 0.01$), detoxification (*gst;* $p < 0.001$), heat shock (*hsp90*; $p < 0.01$), apoptosis (*aif1, ai5*; $p < 0.05$), reproduction (*vtg;* $p < 0.05$) and Cu transport (*ctr1;* $p < 0.05$), were all upregulated, were as metallothionein *mta* was down-regulated ($p<0.5$) (Fig. 6).

3.4 Gene expression changes in neonates

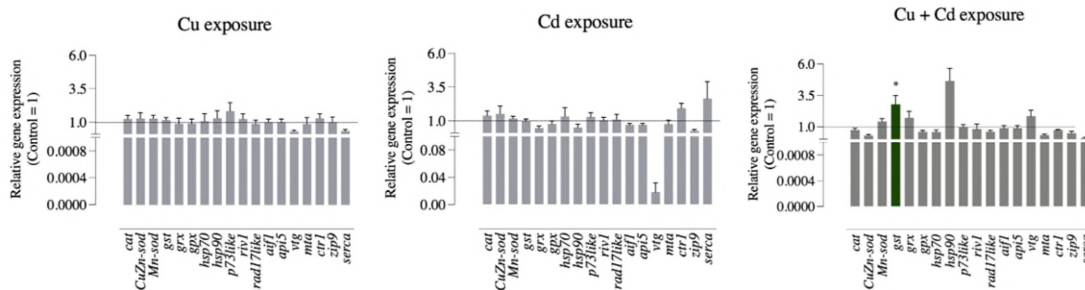
Gene expression was also studied in neonates under all exposure conditions (Fig.7). In neonates, in addition to the genes that were studied in adults, development related genes (*opsin, vri, cp, notch2*) were also studied. Aqueous exposure resulted in upregulation of the antioxidant gene *Mn-sod* ($p < 0.5$) in mixture-exposed animals. In the mixture-exposed animals, *gst,* another antioxidant gene, was also upregulated ($p < 0.05$). In addition, aqueous exposure pathway resulted in upregulation of *hsp90, aif1, api5* and *ctr1 gene* ($p < 0.05$) and down -regulation of metallothionein *(mta).* In the animals exposed via the diet, Cu exposure alone and mixed exposure resulted in upregulation of the calcium transport gene *serca* (*p* <

0.05), whereas downregulation of *CuZn-sod (p < 0.05*), *riv1* (*p < 0.01*), and *vri* (*p < 0.05*) was observed in the mixture exposed animals. In animals exposed via the combined pathway, most genes were upregulated in response to the mixture (*cat*: p < 0.001, *Mn-Sod*: *p < 0.05, gst*: *p < 0.001*, *gpx*: *p < 0.05*, *hsp90*: *p < 0.01*, *p73* like: *p < 0.05*, *aif1*: *p < 0.001*, *vtg*: *p < 0.05, opsin*: *p < 0.05*, *mta* p < 0.001, *serca*: *p < 0.01*). In addition, downregulation of *vri* (*p < 0.05*), *cp* (*p < 0.05*) and *ctr1* (*p < 0.01*) was also observed. Exposure to Cu alone also resulted in downregulation of *cp* (*p < 0.05*), *ctr1* (*p < 0.05*). A common response of mixture exposed animals via all exposure pathways was upregulation of vtg *(p<0.05).*

A Aqueous exposure



B Dietary exposure



C Combined exposure

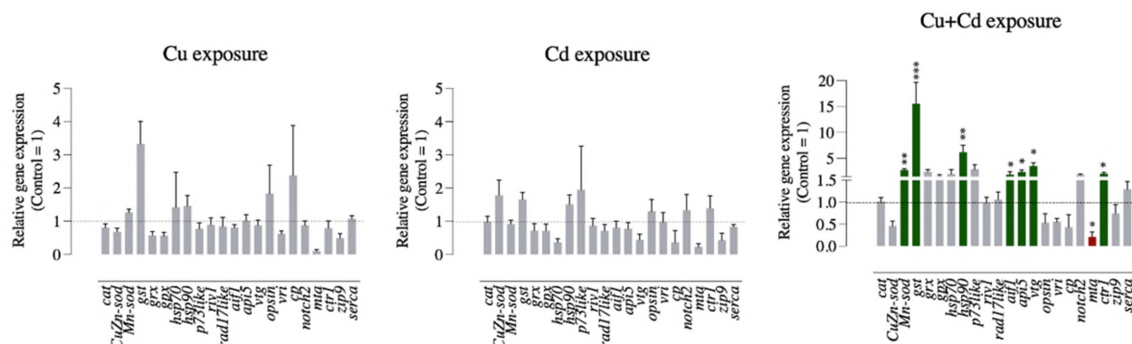
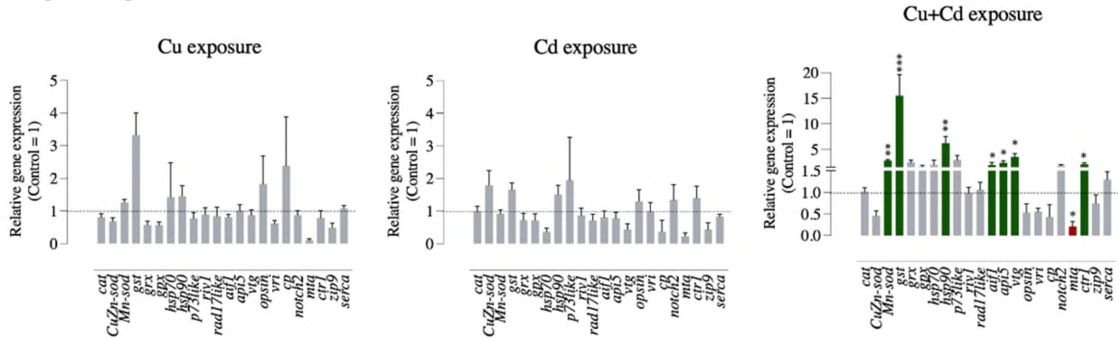
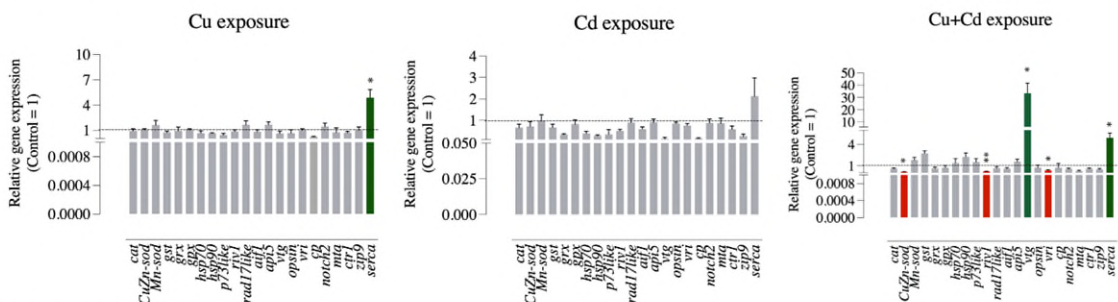


Fig. 6. Molecular responses in *D. magna* adults. Transcript level of genes related to antioxidative, apoptosis, DNA repair, reproduction and metal transport in adults exposed for 7 days to Cu, and Cd alone and a combination via; (A) aqueous (B) dietary (C) combined exposure. The green colour represents up-regulation of genes relative to control. The values indicated in figure are the average ± standard error of

mean (SEM) of minimum 8 biological replicates (pooled data of two independent experiments). A non-parametric test (Kruskal – Wallis and Dunn's multiple comparison test) was used to compare the treatment and the control groups. **p<0.05, **p<0.01, ***p<0.001.*





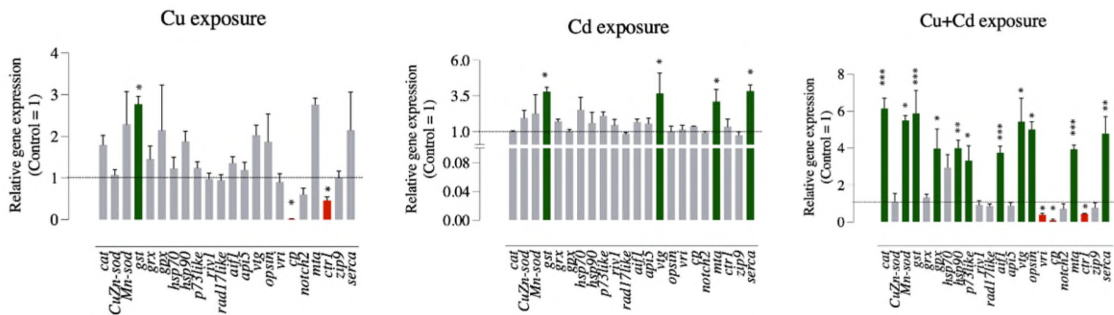


Fig. 7. Molecular responses in *D. magna* neonates. Transcript level of genes related to antioxidative, apoptosis, DNA repair, reproduction, development, and metal transport in neonates exposed for 7 days to Cu, and Cd alone and a combination via; (A) aqueous (B) dietary (C) combined exposure. The green colour represents up-regulation of genes relative to control. The values indicated in figure are the average ± standard error of mean (SEM) of minimum 8 biological replicates (pooled data of two independent experiments). A non-parametric test (Kruskal – Wallis and Dunn's multiple comparison test) was used to compare the treatment and the control groups. **p<0.05, **p<0.01, ***p<0.001.*

3.5 Redox state in adults and neonates

The impact of metal exposures was assessed on the redox state by quantifying the hydrogen peroxide (H_2O_2) and glutathione levels in both adult daphnids and neonates (Fig.8). Exposure to a mixture of Cu and Cd through the combined pathway resulted in a significant increase in H_2O_2 levels in adult animals *($p<0.05$).* No effect on H_2O_2 levels was observed following the exposure through aqueous and dietary exposures. The measurement of the GSH:GSSG ratio showed a decrease in Cu and Cd mixture exposed animals through the combined pathway *($p<0.05$).* Similar results were recorded in neonates exposed to the mixture via the combined pathway. Neonates, unlike adults, exhibited a significant increase in H_2O_2 levels with exposure to Cu and Cd mixture through both aqueous and dietary exposure *($p<0.05$).* A significant decrease was observed in response to Cu and Cd – alone exposed animals via the combined pathway *($p<0.5$).*

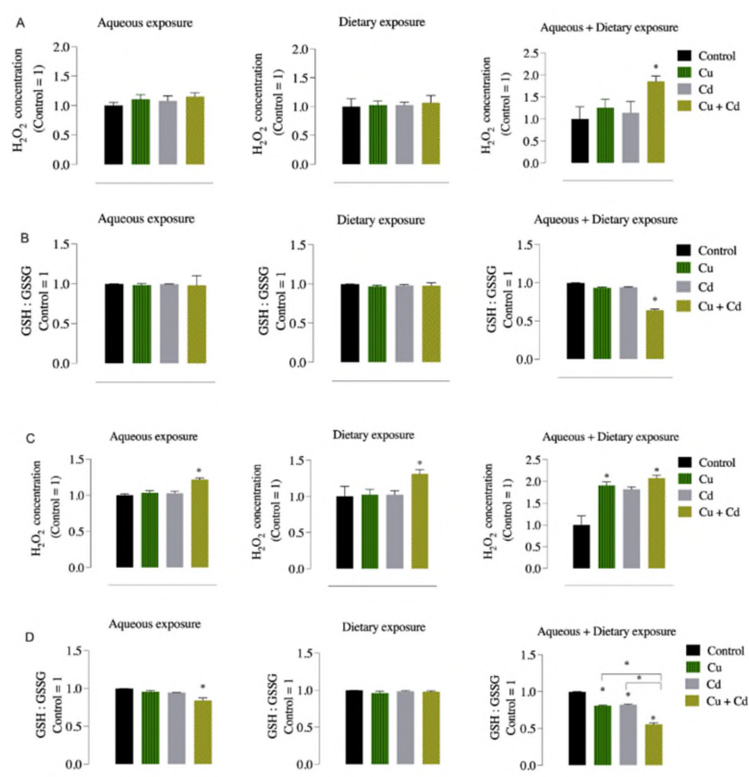


Fig. 8. Redox response in *D.* magna adults and neonates. Graphs showing (A) H_2O_2 levels in adults (B) GSH:GSSG levels in adults (C) H_2O_2 levels in neonates (B) GSH:GSSG levels in neonates (C). The values indicated are the average ± standard error of mean (SEM) of minimum 8 biological replicates. Two independent experiments were performed and the results of two experiments confirm each other. *$p<0.05$

4. Discussion

Although the toxicity of metal mixtures has been extensively studied, age-specific sensitivities to metal mixtures via different exposure pathways has received less attention. More studies have been conducted on the toxic effects of dietary and aqueous exposures separately than on their combination, nevertheless, the combined exposure pathway is the most likely exposure scenario. The present study demonstrates that *D. magna* adults and neonates were more sensitive to the Cu and Cd mixture than to individual exposures to these metals; and combined exposure pathways were found to be more toxic than either aqueous or dietary exposures alone and similar mechanisms were activated. The study also adds to growing evidence that early life stages are more sensitive to metal toxicity than adults.

4.1 Metal accumulation via aqueous, dietary and combined pathways

In adult animals, mixed exposure via all exposure pathways resulted in a decrease in Cd accumulation, indicating an inhibitory effect of Cu on Cd uptake (Fig2. & Fig3). In neonates, the trend of metal accumulation was inconsistent with that in adults. Exposure of neonates via the aqueous exposure resulted in higher Cd concentrations in Cu and Cd mixture condition, whereas the opposite was observed when mixed exposure occurred via the diet or via combined pathways, where Cu inhibited Cd uptake. We found similar results in our previous experiments with planarians (Majid et al., 2022), in which developing and adult animals exhibited different Cd accumulation trends. In other organisms, no consistent results were reported on the interaction between Cd and Cu during uptake. Komjarova and Blust (2008) found an inhibition of Cu uptake by Cd in *D. magna,* while the opposite was found in *Danio rerio* (Komjarova and Blust, 2009a; Komjarova and Bury 2014; Majid et al., 2022, see Chapter 1). Interactions between Cu and Cd occur even at low concentrations (Komjarova and Blust, 2008; Cobbina et al., 2015), through ionic and molecular mimicry at the site of transporters of essential elements and/or molecules (Bridges and Zalups 2005) leading to significant changes in the apparent properties of the components and form complexes that have deleterious effects on organisms (Altenburger et al. 2013). As such, Cd can replace essential metal ions, mainly Zn^{2+}, Cu^{2+} and Ca^{2+} in metalloenzymes (Brzóska and Moniuszko-Jakoniuk 2001) and affect the binding of metal ions to -SH groups of enzymes, proteins and nucleic acids to metal ions. (Zalups, 2000). We further compared the accumulation patterns in aqueous and dietary phases to broaden our understanding of the role of water and diet in the sourcing of Cu and Cd. The adults and neonates exhibited more elevated levels of metal uptake when exposed via aqueous than to diet alone, indicating

water as a significant pathway for metal uptake. These results are consistent with previous studies demonstrating a higher uptake of Cu and/or Cd from water compared to diet (Kadiene et al., 2019). When examining the accumulation patterns of individual pathways of aqueous and dietary exposure and subsequently comparing them with their combined exposure, accumulation patterns of aqueous and combined exposure were comparable, with the exception of one exposure condition, in which neonates, showed increased levels of Cd via the combined pathways. Normally, in combined exposure scenarios, uptake via multiple routes, including ingestion through water, dietary uptake and direct surface adsorption (Kadiene et al., 2019) is expected. However, in the current study, deviation from this expected outcome suggests that factors such as, the differential bioavailability of metals between exposure routes could have influenced the observed accumulation pattern (Mohammad & Bala, 2015). Further investigation of the specific mechanisms underlying the observed variations is warranted to fully understand the dynamics of metal accumulation in combined exposure scenarios. When comparing metal accumulation between adults and neonates, the accumulation was in general lower in neonates in each exposure condition compared to adults. We also had similar results in other tested species (see Chapter 1 and Chapter 3) and can be attributed to the physiological and developmental differences in metal uptake and distribution from the adult animals (Mohammad et al., 2013; Majid et al., 2022).

4.2 Cu and Cd toxicity responses in adults

As illustrated in Figures 4 and 5, the effects on reproductive capacity and the activation of molecular stress responses were more pronounced in adults exposed to the mixture of Cu and Cd through the combined uptake pathway. Exposure to Cu and Cd individually through combined pathways also led to decrease in reproductive capacity. However, in a broader sense, it can inferred that the most potent impact on reproductive capacity, surpassing additive effects resulted from the joint effects of Cu and Cd via the combined pathways. It is challenging to distinguish and characterize which metal in the Cu and Cd mixture was more toxic and which exposure pathway was more important. However, the observed metal accumulation patterns, showing a suppression of Cd uptake in the presence of Cu, as well as the adverse impact of Cu single exposures on reproductive capacity, suggest a potential link between the impaired reproductive capacity and Cu toxicity. This assertion is supported by the upregulation of copper transport protein *ctr1* to facilitate the transport of Cu (Lee et al., 2001; Nardella et al, 2022).

In order to gain insights into molecular events underlying the observed impairment in reproductive capacity, we examined the gene expression patterns of various categories of genes across all experimental groups (see Fig. 6). Previous studies have attributed reduced reproductive capacity to systematic factors (such as impaired digestion) and behavioral responses (such as food avoidance) (Sofyan et al., 2007). These factors may potentially contribute to the observed effects in our study. However, we did not specifically analyse these responses as they were not the primary goal of our study. We attribute reduced reproductive capacity to the accumulation of Cu and Cd at the target sites where the synthesis or processing of reproductive protein vitellogenin *(vtg)* occurs. The gene expression pattern of *vtg* also serves as an indication of this. Vitellogenin serves as the precursor for the gene encoding yolk protein and plays a vital role in the reproductive processes of daphnia (Gust et al., 2021). The quantity of *vtg* is a key determinant of oocyte size and quantity, as indicated by previous studies (Jubeaux et al. 2012; Gust et al., 2021). Studies have reported both the induction and suppression of *vtg* in response to diverse range of chemicals (Hannas et al., 2011; Samanta et al., 2020). Upon analysis of the expression pattern of *vtg* gene in adults, a pathway dependent pattern was observed. The *vtg* gene exhibited increased expression mainly in response to Cu and Cd mixture through combined exposure pathway affecting reproductive mechanism. We associate the increased *vtg* activity to counteracting the loss of protein function. This compensation is achieved through increasing *vtg* receptor proteins to facilitate the restoration of oocyte growth (Morini et al., 2020). Alternatively, it may be linked to the defense mechanism employed to counteract the oxidative stress, a condition characterised by an imbalance in the redox state i.e., between oxidants and antioxidant capacity within a cell (Seehuus et al., 2006; Veltman et al., 2023). To further validate this finding, we evaluated the transcriptional profile of various antioxidant genes as depicted in Figure 6. The expression levels of antioxidative enzyme genes specifically manganese superoxide dismutase (*Mn-sod),* and glutathione S-transferase (*gst)* were both upregulated in the Cu and Cd mixture exposed group via the combined exposure pathway. The activity of *gst* was also altered in the animals that were exposed to Cu and Cd mixture via diet. However, the effect was more pronounced in the combined setup. We suggest that the upregulation of *gst* served as a protective mechanism against oxidative stress and the presence of free metals due to the ability of glutathione to bind to the metals and isolate the resulting ligand-metal complex as suggested by previous studies (Cobbett, 2000; Jozefczak et al., 2012; Presnell et al., 2013; Gasmi et al., 2022). In

addition, the upregulation of *hsp90* signifies a robust defensive response against oxidative damage resulting from the additive effects of Cu and Cd on reproduction together with the additional influence of exposure to these metals via aqueous and dietary pathways (Jackson, 2013; Ikwegbue et al., 2018).

In order to further validate the observed redox alterations at molecular level, we evaluated hydrogen peroxide (H_2O_2) and glutathione (GSH) as a marker of cellular toxicity. Hydrogen peroxide is a central redox signalling molecule, capable of serving as messenger to carry a redox signal from the site of its generation to a target site (Sies, 2017). The levels of H_2O_2 were found to be increased in animals exposed to the metal mixture via the combined pathway (Fig. 7). Furthermore, reduced glutathione (GSH) is widely recognised as an important scavengers of reactive oxygen species (ROS), and its ratio with oxidised glutathione (GSSG) can serve as a marker of oxidative stress (Zitka et al., 2012). The GSH:GSSG ratio were solely reduced in the same experimental groups that exhibited elevated levels of H_2O_2. Overall, these results show that the co-exposure to Cu and Cd via combined exposure pathway stronger effect on reproductive capacity which was likely attributed to oxidative stress.

4.3 Cu and Cd toxicity responses in Neonates

As in the adult animals, the effects of Cu and Cd mixture through combined pathway were more pronounced in the neonates. A decrease in survival was only observed in neonates when exposed to both Cu and Cd via the combined pathway, indicating higher stress in the neonates. This increased stress in neonates may be attributed to their higher sensitivity due to the early life stage, as observed in previous studies (Hoang et al., 2004; Hoang and Klaine, 2007; Mohammad, 2013; Wise, 2022). The increased sensitivity can also explained by the differences in their morphological, physiological, and biochemical characteristics as emphasised by Mohammad (2013).

To understand the molecular events underlying the observed effects on the growth of neonates, we examined the transcriptional profile of the same sets of genes that were examined in adults across all exposure groups (see Fig. 7). In addition, genes related to developmental process were studied in neonates. Vitellogenin is important for endocrine regulation, including development and reproduction. It is known that *vtg* genes are directly expressed in response to environmental stressors (Hannas et al., 2011; Samanta et al.,

2020). Some insects have been found to exhibit an increase in the expression of *vtg* during the early stages of development as described by Shu et al., (2009) and Chen et al., (2022). We analysed the expression pattern of *vtg* gene in neonates to assess the effect on ovarian development. The exposure to a mixture of Cu and Cd caused induction of *vtg* gene under all the three exposure setups, i.e., aqueous, dietary and combined pathway. The combined pathway also lead to the up-regulation of *vtg* expression when exposure occurred via Cd only. This elevation of *vtg* activity may be a protective mechanism to counteract the toxicity of metal exposure and oxidative stress (Havukainen, et al., 2013).

To determine whether the observed effects on neonates exposed were primarily driven by metal toxicity or represented physiological responses associated with molting, we analysed the transcriptional activity of cuticular protein (*cp*). Cuticular proteins are crucial components of the exoskeleton in arthropods, including Daphnia, and play a significant role in the moulting process (Tan et al., 2022). During normal molting, the upregulation of *cp* promotes molting cycle completion and ensures the proper growth and development of crustaceans (Giraudo et al., 2017). The downregulation of *cp* expression observed in Cu-exposed neonates, as well as those exposed to mixture via the combined pathway, indicate potential interference with normal molting processes, likely due to the toxic effects of Cu exposure rather than a typical physiological response during molting. Underlying to these morphological effects, we noticed a down regulation of the copper transport gene *ctr1* during combined exposure and can be linked to counter toxic accumulation of Cu. This assumption is supported by observed upregulation of *mta,* indicating a potential mechanism for sequestration and detoxification of excess Cu ions. In neonates, besides *vtg,* there were several genes than adults that exhibited altered expression, which is in correspondence to the physiological observations. Moreover, more similarities between exposure pathways were observed. Two genes showed a similar expression during dietary exposure and exposure via combined pathway, which are v*ri*, which was downregulated and *serca,* a calcium transport protein, which was upregulated. A mixed exposure led to an upregulation of the antioxidative genes *Mn-sod, gst, gpx* and *opsin*, genes involved in eye development, when exposed via the aqueous pathway or via a combination of both pathways. The expression of none of these genes was altered during dietary exposure. The upregulation of antioxidant genes suggests a strong response against oxidative damage due to the interactive effects of Cu and Cd as well as the contributing effects of both the aqueous and dietary exposures. In addition, the upregulation of the heat shock protein *hsp90* which

occurred only upon combined exposure to Cu and Cd, could be a response to stress for maintaining cellular homeostasis (Jackson, 2013).

As in adults, to further validate the redox alterations we evaluated H_2O_2 and GSH at cellular level. The levels of H_2O_2 were found to be increased in animals exposed to the metal mixture via the combined pathway (Fig. 8). This experimental group also exhibited a decrease in GSH:GSSG ratio. In neonates, metallothionein was also upregulated during exposure to Cd via the combined pathway, which may be due to its strong affinity for Cd or is to counteract the damage due to metal toxicity (Wang et al., 2014; Wang et al., 2018; Bakiu et al., 2022). Overall, the results show that (1) Cu and/or Cd toxicity is increased by the simultaneous presence of another metal, and due to interactive effects of the two metals (more than additive) (2) neonates are more sensitive to metal toxicity than adults, at least in the combined exposure scenario, and (3) exposure pathways are critical for toxicity and that oxidative stress is the common mechanism underlying toxicity.

Conclusion

Our study provides valuable insights into the toxicity of Cu and Cd in a single and co-exposure scenario. Our results show that the toxicity of Cu and/or Cd is significantly increased in the presence of another metal, which emphasises the importance of considering interactive effects when assessing the toxicity of metals. Furthermore, the observed effects of the co-exposure indicate synergistic interactions between Cu and Cd. We found that neonates are more susceptible to metal toxicity compared to adults, particularly in the context of combined exposure scenarios. This increased susceptibility emphasises the importance of considering age-related differences when assessing toxicity. In addition, our study emphasises the critical role of exposure pathways in determining metal toxicity. Underlying the toxicological outcomes in both adults and neonates, oxidative stress was found as a common mechanism underlying the toxicity of these metals. However, it is important to recognise that our research focused primarily on two metals (Cu and Cd) and their combined toxicity. However, environmental exposure scenarios often involve complex mixtures of metals and other pollutants. Future research should investigate the toxicological effects of metal mixtures to better reflect real-world environmental conditions and enable more comprehensive toxicological assessments. Finally, while our study sheds light on the importance of exposure pathways in metal toxicity, further research is needed to elucidate

the specific mechanisms underlying differential toxicity across exposure pathways in multiple species.

Supplementary data

Table S1: Supplementary data

Accession number and nucleotide sequences of genes obtained from NCBI database

Gene	Abbreviation	Accession no.	Forward primer	Reverse primer
Actin*	*act*	AJ292554	CCA CAC TGT CCC CAT TTA TGA A	CGC GAC CAG CCA AAT CC
18S ribosomal RNA*	*18s*	AF070104	CGC TCT GAA TCA AGG GTG TT	TGT CCG ACC GTG AAG AGA GT
28S ribosomal RNA*	*28s*	AF532883	GAG GCG CAA TGA AAG TGA AG	TGT TCG AGA CGG GAT CA
β-Actin*	β-*act*	AJ292554.1	CCA CAC TGT CCC CAT TTA TGA A	CGC GAC CAG CCA AAT CC
glutathione peroxidase	gpx	KX358698.1	CGTGGCTACTTACTGAGGGTTT	CGGACGAACGTAACGGATT
Heat shock 70	*hsp70*	EU514494	ACTGATGCCGTGATTACTGTTC	CCTTGTGATGCTGGTGTAGAA
Heat shock 90	*hsp90*	KX358700	CCGAGGAAGAGAAACCAAAG	CGTCGACCGAATACTTCTCC
Vitellogenin	vtg	AB252737.1	CTG TTC CTC GCT CTG TCT TG	CCA GAG AAG GAA GCG TTG TAG
Opsin	Opsin	DW724560	TCCTCGTGCTTGAAGAC	GCGCTTGTTTCGGATAC
Metallothionein - a	mt-a	DV437799	TTGCCAAAACAATT GCTCAT	CACCTCCAGTGGC ACAAAT

*Housekeeping genes (HKG)

Supplementary Table S2. MIQE guidelines concerning qPCR experiment.	
Experimental design	
Definition of experimental and control groups	Experimental groups - *D. magna* adults (20-21 days old): 7 days exposure to 0.25 µM Cu, 0.02 µM Cd singly and as a co-exposure via aqueous exposure. - *D. magna* adults (20-21 days old): 7 days exposure to 4.33 x 10^3 ng dw Cu/daphnid, 5.72 x 10^1 ng dw Cd/daphnid and a combination of 4.33 x 10^3 ng dw Cu/daphnid and 5.72 x 10^1 ng dw Cd/daphnid via dietary medium. - *D. magna* adults (20-21 days old): 7 days exposure to Cu Cd and Cu+Cd via aqueous + dietary exposure. - Control groups: *D. magna* adults in plain medium - *D. magna* neonates (24 h old): 7 days exposure to 0.25 µM Cu, 0.02 µM Cd singly and as a co-exposure via aqueous exposure. - *D. magna* neonates (24 h old): 7 days exposure to 4.33 x 10^3 ng dw Cu/daphnid, 5.72 x 10^1 ng dw Cd/daphnid and a combination of 4.33 x 10^3 ng dw Cu/daphnid and 5.72 x 10^1 ng dw Cd/daphnid via dietary medium. - *D. magna* neonates (24 h old): 7 days exposure to Cu, Cd and Cu+Cd via aqueous + dietary exposure. - Control groups: *D. magna* neonates in plain medium.
Number within each group	$n \geq 8$
Sample	
Procedure and / or instrumentation	Frozen animals were disrupted by chemical lysis in 200µl RNA lysis/binding buffer (Qiagen, catalogue number 79216). RNA was isolated using a phenol-chloroform extraction procedure and was precipitated with Na-acetate and 70% ethanol and resuspended in RNase – free water.
Details of DNase or RNase treatment	Genomic DNA was removed with the Turbo DNA free kit (Ambion® Thermo Fisher Scientific).
Nucleic acid quantification	Nucleotide concentrations were assessed on the Nanodrop ND-1000 spectrophotometer (NanoDrop® ND-1000, ISOGEN Life Science).
Purity	260/280 and 260/230 analysis
Reverse transcription	
Complete reaction conditions	cDNA was synthesized using Superscript TM III first-strand synthesis supermix (Thermo-fisher Scientific)
Amount of RNA and reaction	200ng of total RNA in a reaction volume of 20 µl
Storage condition of cDNA	cDNA was diluted to 1:9 in molecular water before storage at -20 °C
qPCR protocol	
Complete reaction conditions	SYBR Green Master Mix (Applied Biosystems)
Reaction volume and amount of cDNA	Reaction volume: 10 µl cDNA: 2.5 µl
Primer	0.3mM of forward and reverse primer
Polymerase, Mg2+. dNTP buffer	Included in SYBR Green Master Mix (Applied Biosystems, Thermo Fisher Scientific, US)
Complete thermocycling parameters	Universal cycling conditions: 10 minutes at 95^0 C 40 cycles: 15s at 95^0 C and 60s at 60^0 C
Manufacturer of qPCR instrument	ABI PRISM 7500 (Applied Biosystems)
qPCR validation	
Specificity (gel, sequence, melt or digest)	Samples with a melt temperature T_m deviating from the product specific T_m were excluded.
For SYBR Green I, Cq of the NTC	NTC's gave no amplification or at least 5 cycles higher than the highest sample Ct value
PCR efficiency	0.85 – 1.15
R^2 of calibration curve	≥ 97%
Data analysis	
qPCR analysis program (source)	qBase (Biogazelle)
Method of Cq determination	Hellemans et al., 2007
Results for NTCs	NTC's gave no amplification or at least 5 cycles higher than the highest sample Ct value
Justification of number and choice of reference genes	geNorm analysis
Statistical method for results	Non-parametric, Kruskal – Wallis test
Software (source, version)	Prism GraphPad 8.1.2 (GraphPad software, CA, US).

Chapter 3

Planarian *Schmidtea mediterranea*

Chapter 3. Interactive toxicity of copper and cadmium in regenerating and adult planarians

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Abstract

In a polluted environment, metals are present as complex mixtures. As a result, organisms are exposed to different metals at the same time, which affects both metal-specific as well as overall toxicity. Detailed information about the molecular mechanisms underlying the adverse effects of combined exposures remains limited in terms of different life stages. In this study, the freshwater planarian *Schmidtea mediterranea* was used to investigate developmental and physiological responses associated with a combined exposure to Cu and Cd. In addition, the cellular and molecular mechanisms underlying the provoked adverse effects were studied in different exposure scenarios. Mixed exposure resulted in a decline in survival, diverse non-lethal morphological changes, neuroregenerative impairments, altered behaviour and a limited repair capacity. Underlying to these effects, the cellular redox state was altered in all exposure conditions. In adult animals, this led to DNA damage and corresponding transcriptional changes in cell cycle and DNA repair genes. In regenerating animals, changes in hydrogen peroxide and glutathione contents led to regenerative defects. Overall, our results demonstrate that (1) developing organisms are more susceptible to metal exposures, and (2) the toxicity of an individual metal increases significantly in a mixed exposure scenario. These aspects have to be included in current risk assessment strategies.

1. Introduction

Due to widespread anthropogenic activity, metal pollution remains an important threat to our environment. Their persistence leads to an accumulation in various compartments (ATSDR, 2004; 2012), resulting in a concomitant exposure and a growing concern about the adverse effects of metal mixtures (Kamunde and MacPhail, 2011; Tchounwou et al., 2012; Anyanwu et al., 2018; Alidadi et al., 2019; Briffa et al., 2020; Balali-Mood et al., 2021). Despite this growing interest, our mechanistic understanding of the processes that lead to visible effects in growth and development is still scarce.

Copper is an essential micronutrient that is involved in various biochemical processes. It is required for embryonic development, mitochondrial respiration, hemoglobin production and neural functions (Chen et al., 2008; Rivera-Mancia et al., 2010; Vetchý, 2018). Copper is a cofactor of redox enzymes such as superoxide dismutase (Cu/Zn SOD), cytochrome oxidase and ceruloplasmin, and is involved in blood clotting as a part of Factor IV and in the synthesis of thyroxine, a thyroid hor- mone (Kim et al., 2008; Osredkar and Sustar, 2011; Guengerich, 2018). While Cu deficiency causes adverse developmental and neurobehavioral effects (Hefnawy and El-khaiat, 2015), increased levels of Cu result in tissue damage and diseases such as Menke's and Wilson's disease (Bleackley and Macgillivray, 2011; de Romaña et al., 2011; Taylor et al., 2020). Several mechanisms have been proposed to describe Cu-induced toxicity. Most often, the onset of Cu toxicity is linked to the ability of Cu to undergo redox cycling from oxidised (Cu2+) to reduced (Cu+) forms, catalyzing the formation of reactive oxygen species (ROS) via the Haber-Weiss reaction (Guecheva et al., 2001; Manzl et al., 2004; Stern et al., 2007; Driessnack et al., 2017). ROS can attack DNA and proteins, or can induce lipid peroxidation, leading to an altered membrane fluidity and permeability (Mattie and Freedman, 2001 Gaetke et al., 2014; Lesiów et al., 2019).

Cadmium is a biologically nonessential metal. It is classified as a Group − 1 human carcinogen by the World Health Organisation's International Agency for Research on Cancer (IARC, 1993), and a 1B carcinogen by the European Chemicals Agency (ATSDR, 2012; ECHA, 2013). At the physiological level, Cd interferes with the morphology and/or function of respiratory, urinary, cardiovascular, gastrointestinal, immune and nervous systems (Lippmann, 2000; Risso-de-faverney 2001; Filipič, 2012; Satarug et al. 2017, Satarug, 2018). Along with its teratogenic and mutagenic effects, Cd affects both male and female reproductive systems (Kumar and Sharma, 2019). At the cellular level, Cd replaces essential co-factors or binds to

specific groups such as thiol groups. As a result, ROS are produced through the displacement of the endogenous Fenton metals from enzymes such as catalase or superoxide dismutase (Mn SOD and Cu/Zn SOD) (Cuypers et al., 2010; Nair et al., 2013). Increased oxidative stress leads to mitochondrial dysfunction, cellular glutathione depletion and lipid peroxidation (Nair et al., 2013; Branca et al., 2020). In addition, Cd indirectly provokes DNA damage, leading to changes in cell cycle dynamics (Zarros, 2008) Rani et al., 2014; Genchi et al., 2020).

Reported effects on early life after a combined exposure to Cu and Cd include decreased ingestion rates, decreased reproduction, delayed maturation, reduced body size, and lowered somatic growth rate in *Daphnia pulex* (Sadeq and Beckerman, 2020). In this study, we further explored how a combined exposure to Cu and Cd affects developing tissues, using regenerating planarians as a proxy. The unique characteristics of planarians such as their high regeneration potential based on the presence of pluripotent stem cells, and a well-organized nervous system that they can fully regenerate (Oviedo et al., 2008, Hagstrom et al., 2015, 2016) make them an interesting model system to study develop- mental toxicity. Regenerating planarians can be considered as developing planarians, that restore/repair their missing body parts after they have been amputated to create individual head and tail fragments. In the past, several studies have reported morphological, locomotive and behavioural impairments of planarians after exposure to Cu and/or Cd (Guecheva et al., 2001, 2003; Knakievicz and Ferreira, 2008; Plusquin et al., 2012; Zhang et al., 2014; Van Roten et al., 2018; Sunardi et al., 2020). Knakievicz and Ferreira (2008) demonstrated a significant effect on the mobility and time of regeneration in the planarian *G. tigrina* after exposure to low levels of Cu (0.10 mg L^{-1} = 0.40 µM). A significant reduction in reproductive performance was also demonstrated. Another study showed a disruption in the growth and regeneration of *Dugesia tigrina* in response to Cu levels of 0.009–0.15 ppm (0.04–0.60 µM) (Sunardi et al., 2020). Studies by Wu et al. (2014, 2015) on *Dugesia japonica* have shown a disturbance of the neurotransmission system following Cd exposure. In addition, we previously demonstrated an altered cell cycle dynamics in response to Cd intoxification in planarian *Schmidtea mediterranea* (Plusquin et al., 2012). Although, the toxicity of Cu and Cd exposures has been extensively studied in a single exposure context, studies addressing the developmental toxicity of these metals in a mixture scenario remains largely unanswered. Nonetheless, a thorough understanding of the impact of metal mixtures on development is essential for future risk analysis.

In this study, we studied lethal and sublethal endpoints in regenerating and adult animals, in relation to their internal metal accumulation. Second, we identified the underlying cellular and/or molecular mechanisms and assessed the recovery potential of developing animals. Understanding such interactions not only enables a better understanding and assessment of metals as toxicants, but also allows an assessment of the susceptibility of developing and adult species.

2. Material and method

2.1 Experimental design

Asexual strains of the freshwater planarian *Schmidtea mediterranea* (4–5 mm in length) were cultivated in a culture medium consisting of 1.6 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.1 mM $MgCl_2$, 0.1 mM KCl and 1.2 mM $NaHCO_3$ in Milli-Q water (Pirotte et al., 2015). The animals were kept in dark at 19–20 ^{0}C and were fed veal liver once a week, which was kept at − 20 ^{0}C. After 4 h of feeding, the remaining liver residue was removed, the animals were rinsed twice with distilled water and fresh medium was added. Feeding was stopped at least 7 days prior to exposure to avoid food-related effects (Baguna, 1974) and ambiguity in the route of exposure. Four exposure conditions, comprising a control (freshwater medium), copper in the form of $CuCl_2{\cdot}2H_2O$ (Merck - Darmstadt, Germany), cadmium in the form of $CdCl_2.2.5H_2O$ (Sigma – Aldrich, St. Louis, MO, USA), separately and in a mixture were chosen. One Molar $CuCl_2$ and $CdCl_2$ stock solutions were prepared in ultra-pure water (Milli-Q, Bedford, MA, USA) and freshly diluted to 20 µM $CuCl_2$, 10 µM $CdCl_2$ and to the mixture of 20 µM $CuCl_2$ and 10 µM $CdCl_2$ in freshwater medium (prepared from ultrapure water) before use. During the experiments, the animals were exposed in sterile 24-well plates with one animal per well in 1 ml of medium. Exposure solutions were renewed every 2–3 days and the control animals were kept in freshwater medium and were refreshed simultaneously. Old solutions were carefully removed with a pipette (leaving some solution behind) without harming the animals and fresh solutions (1 ml) was added in each well. Samples of test media were filtered through a 0.22 µM filter, acidified with 200 µL of ultra-pure nitric acid and analysed for dissolved Cu and Cd using inductively coupled plasma-mass spectrometry (7700x ICP-MS, Agilent Technologies). The measured total Cu and Cd concentrations were 95–100% of the desired nominal concentrations (Cu; 19.35 µM, Cd; 9.96 µM, Mixture; Cu 19.27 µM and Cd 9.84 µM). The concentrations of these metals in the control medium were below the method quantifi- cation limit (less than 1 µg/L).

To determine the exposure range, a lethality test of Cu with concentrations between 0 and 120 µM was carried out at five time points (24 h, 48 h, 72 h, 1 and, 2 weeks). Ten animals were used per condition in a blinded and randomised experimental design. Mortality was recorded at different time points, with a rating of zero for the dead and one for living animals. Based on the sub-lethal values of one week (25% of the LC_{50} (lethal concentration 50) at one week), a fixed concentration of 20 µM (95% confidence interval) was selected for Cu. For Cd, a concentration of 10 µM (sub-lethal) was chosen, based on the value of previous lethality test results (25% of the LC_{50} for one week) (Plusquin et al., 2012). All further experiments were performed on regenerating (developing) and homeostatic (adult) planarians. To induce regeneration, animals were cut transversally just above the pharynx and exposed over a period of 7 and/or 14 days to 20 µM $CuCl_2 \cdot 2H_2O$, 10 µM $CdCl_2.2.5H_2O$ separately or as a mixture. In regenerating animals, the exposure started 30 min after amputation to allow wound closure, so that the direct uptake of metals via the open wound could be prevented. In contrast, the adult planarians were directly exposed to the respective exposure media for 7 and/or 14 days.

2.2 Metal accumulation

Regenerating and adult animals were sampled after 7 and/or 14 days of exposure and snap-frozen in liquid nitrogen for metal analysis. The tissues were thawed and dried at 60 ^{0}C for 24 h. After drying, the tissues were weighed and digested in 150 µL ultra-pure nitric acid (Trace metalgrade - Merck, Darmstadt, Germany) by microwave-assisted digestion at 100W(3×3minutes),180W(3×3minutes)and300W(1min). Digestates were diluted up to 4 ml with ultra-pure water (Milli-Q, Bedford, MA, USA). The Cu and Cd concentrations of the samples and three blanks were determined by inductively coupled plasma-mass spectrometry (7700x ICP-MS, Agilent Technologies). The concentration was expressed as µg $gdw^{-}1$ (dry tissue weight). A reference standard mussel tissue (NIST - 2976) was used to assess the validity of the protocol.

2.3 Morphology, growth, and survival

Morphological changes were analysed to evaluate the effects of single and mixture treatments of Cu and Cd on the morphology, growth, and survival of regenerating and adult animals. Over a period of 14 days, the animals were exposed to control medium (no added metals), 20 µM $CuCl_2$ and 10 µM $CdCl_2$ separately and in a mixture. In both regenerating and adult animals all types of visible malformations were carefully observed and recorded based

on previously studied morphological de- viations in planarians (such as tissue lesions, tissue lysis, blisters, bloating, contracted body, tissue outgrowths, lack of photoreceptors, tissue regression) in response to other stressors (Pirotte et al., 2015; Hagstrom et al., 2016; Van Huizen et al., 2017; Van Roten et al., 2018; Wouters et al., 2020).

Regeneration was determined by measuring the size of the newly formed unpigmented bud at the wound site called a blastema, which is generally formed within 2–3 days of amputation. The size of blastema was quantified using the ImageJ software (version 1.51, National In- stitutes of Health, Bethesda, MD, USA) and expressed relative to the total area of three independent measurements, normalised against the total body area of the animal. Within a span of seven days, the regenerating anterior fragment of the amputated animal forms to a tail with a cen- trally – located pharynx and the regenerating posterior fragment re- constructs a head with two photoreceptors. The regenerative success was assessed by scoring the process as "complete" or "aberrant" based on the reappearance of missing body structures (Wouters et al., 2020). Overall growth was assessed in both regenerating and adult animals by measuring the average worm size at 7 and 14 days post exposure. Digital images were obtained using a CCD camera (DFK 41AF02 FC, The Imaging Source, Bremen, Germany) mounted on a trinocular stereo mi- croscope (Nikon, SMZ 800, Tokyo, Japan). At each observation, dead animals were removed and counted in each exposure medium to monitor mortality. After removing the dead animal the well was emptied and labelled as "empty" in order to avoid miscalculation.

To determine the recovery potential of exposed animals, homeostatic worms were exposed to 0 µM, 20 µM $CuCl_2$, 10 µM $CdCl_2$ separately and in a mixture for a period of 7 days. After seven days of exposure, all animals were transversally amputated above the pharynx in order to obtain a head and tail fragment. The animals were further divided into two batches. One batch of four experimental conditions was allowed to recover in metal-free control (freshwater) medium and the other batch of four groups was continuously exposed to the respective exposure conditions for 14 days. All the animals were screened daily for morphological abnormalities. After 14 days, the regeneration and growth were measured by the above mentioned method.

2.4. Neurophysiology and neurodevelopment

2.4.1 Locomotor velocity

The motility was tested by measuring the locomotor activity in a blinded and randomised experimental set-up. The motility test was modified from the locomotor activity set-up of Raffa et al. (2001a, 2008). Individual animals were placed in a Petri dish containing cultivation or exposure medium, which was then placed above a grid (0.25 cm^2 squares). A cold light source was placed approximately 18 cm above the animals. After a period of 1 min, the number of lines crossed or re-crossed per minute was counted manually during four consecutive minutes.

2.4.2 Anti – synorf 1 immunohistochemistry

To analyse differences in the regeneration of the central nervous system (CNS), immunostaining was carried out using Mouse Anti – synapsin 3C11 (Developmental Studies Hybridoma Bank, 1:50) (Klagges et al., 1996; Cebria, 2007, 2008). Alexa 488-conjugated goat anti-mouse (Molecular Probes, dilution 1:400) was used as the secondary antibody. After exposure to different metal conditions, the animals were fixed on day 7 of regeneration. First the animals were sacrificed, and the mucus layer was removed by immersion in 2% hydrochloric acid (HCl) (MilliQ water) for 5 min on ice. This was followed by a 0.3% Phosphate-Buffered Saline with Triton-X-100 (PBST) wash by gently shaking for 10 min. Next, they were fixed in 4% formaldehyde (in 0.3% PBST) for 15 min at room temperature (RT), washed 2 × 10 min in PBST and bleached overnight at RT in 6% hydrogen peroxide (H2O2) (in PBST). Subsequently, the planarians were rinsed 2 × 10 min in 0.3% PBST followed by blocking of the non-specific binding sites by incubation in 1% Bovine Serum Albumine (BSA) (in PBST blocking solution) for 4 h at 4 ^{0}C. After a wash step with 0.3% PBST for 10 min at RT, they were incubated overnight at 4^{0}C with the primary antibody 3C11 (1:50, blocking solution). The animals were rinsed for at least eight times for 10 min at RT in 0.3% PBST, followed by incubation in blocking solution for 1 h at RT. Next, they were incubated with the secondary antibody, Alexa 488-conjugated goat anti-mouse (1:400, blocking solution), overnight at 4 ^{0}C The secondary antibody was washed off with 0.3% PBST for at least 6 × 10 min and mounted with Immu–Mount (Thermo ScientificTM, 9,990, 402) with ventral side upwards to make the nervous system visible. The samples were studied using a Nikon Eclipse 80i fluorescence microscope (Nikon Instruments, Melville, NY, USA). The cephalic brain ganglia widths were calculated as the ratio of the brain width relative to head width (Hagstrom et al., 2015).

2.5. Cell cycle responses

2.5.1 Anti-phospho-Histone H3 immunohistochemistry

The mitotic activity of stem cells was assessed by immunostaining with anti-phospho-histone H3 (H3 Serine 10), biotin conjugate (Merck Millipore, Billerica, MA, USA, catalogue number 16–189) (Guo et al., 2006). The primary antibody against phosphorylated Histone-H3 (Ser 10) (D2C8, rabbit mAb, Cell-Signalling, Danvers, MA, USA, Cat. No. 3377S, diluted 1:1000) was used, together with the secondary anti-rabbit-IgG Alexa Fluor 568-conjugated antibody (Thermo Fisher Scientific, Cat. No. A-11036, diluted 1:500, 3h at room temperature). The specimens were fixed and stained as previously described by Plusquin et al. (2012) and carefully mounted with Immu–Mount (Thermo ScientificTM, 9,990,402) to prevent dust and entrapment of air bubbles. The total number of mitotic stem cells/mm2 was determined using NIS–Br software (Nikon Instruments, Melville, NY, USA) and normalised to the body size of the animals, which was determined post fixation. The images were processed and quantified using ImageJ software (version 1.51, National Institutes of Health, Bethesda, MD, USA).

2.5.2. Terminal deoxynucleotidyl transferase dUTP nick end labelling assay (TUNEL)

Apoptotic DNA fragmentation was determined using the ApopTag Red In-Situ Apoptosis Detection Kit (Merck Millipore, Billerica, MA, USA) according to the manufacturer's instructions with some modifi- cations based on Pellettieri et al. (2010) and Almuedo-Castillo et al. (2014). Briefly, after exposure, the animals were fixed and per- meabilised using 5% N-acetyl cysteine diluted in Phosphate-buffered saline (PBS) (10 min, RT) and 4% formalin in PBST (0.3% Triton X-100, 20 min, RT). The samples were washed in PBST (5 min, RT) and transferred to 1% Sodium –Dodecyl – Sulfate (SDS) dissolved in PBS (20 min, RT). Two additional washing steps using PBST were performed before bleaching the worms in 6% H2O2 in PBST (overnight, RT). After bleaching, the samples were washed once with PBST (5 min, RT) and once with PBS (5 min, RT) and exposed to 20 µl terminal transferase (TdT) enzyme diluted with reaction buffer (30% TdT enzyme/70% re- action buffer, 4 h at 37 ◦C). After incubation, the samples were rinsed twice using a stop/wash buffer (1 ml stop/wash buffer in 34 ml molecular H_2O, 5 min, RT) and 0.25% BSA - PBST (3 × 5 min, RT). The samples were then placed in 20 µl anti-digoxigenin-rhodamine diluted in blocking solution (47% anti-DIG-rhodamine/53% blocking solution) and incubated at 4 ^{0}C overnight. Finally, the samples were washed using PBST- BSA (0.25%, 6 × 10 min) and mounted with Immu–Mount (Thermo ScientificTM, 9,990,402). To determine the number of apoptotic cells/mm^2, the total

number of fluorescent cells was normalised to the body size of the animals, which was determined by measuring the sur- face of the animal post fixation using the ImageJ software (version 1.51, National Institutes of Health, Bethesda, MD, USA).

2.5.3 Fluorescence in situ hybridization (FISH)

Whole–mount fluorescence in situ hybridization was performed to assess the differentiation of neoblast gene *NB.21.11e* (the early neoblast progeny marker that is committed to the epidermal cell lineage). DIG –labelled RNA probes were synthesised using the DIG RNA labelling kit (Sp6/T7) (Sigma-Aldrich Cat. No. 11175025910) according to the manufacturer's instructions. *NB.21.11e* primers forward: 5′ GTGATTGCGTTCGCGTATATT 3′ , reverse: 5′ ATTTATCCAGCGCGTCA TATTC 3′ were used. The fluorescent in situ hybridization was performed based on protocols previously described (King and Newmark, 2013; Currie et al., 2016). Briefly, the mucus was removed with 5% N-acetyl cysteine in PBS (8 min) and the animals were fixed in 4% formaldehyde/PBS (20 min). The samples were bleached with form- amide solution (5% non – deionized formamide, 0.5x, 1.2% H_2O_2) under direct light for 2 h. The samples were subsequently washed with 1X SSC once and twice with PBSTx (5 min each). After rehydration and per- meabilisation of the tissue with 20 µl proteinase K (5 min, RT), the samples were immediately transferred to a 4% formaldehyde/PBST so- lution for post fixation. This was followed by two PBST washing steps (5 min each) and prehybridization step. First the samples were incubated for 5 min in a 1:1 solution of prehyb and PBST, and then were incubated in prehybridization buffer (50% deionized formamide, 5X SSC, 0.1 mg/ml yeast RNA, 1% Tween 20 in molecular water) for 2 h at 56 ^{0}C. The probe was diluted in hyb solution (prehybridization buffer + 5% dextran sulfate) in a concentration range of 1–5 ng/µl. After this the probe was heated to 80 ^{0}C for 5 min and quickly cooled on ice during 2 min prior to adding. The Hybridization took place overnight (at least 18 h) at 56 ^{0}C with gentle shaking. An equal amount of 2X SSC/0.1% Triton-X-100 was added and incubated for 20 min at 56 ^{0}C. The samples were then washed thrice with 2X SSC/0.1% Triton-X-100 at 56 ^{0}C for 20 min each; then four times with 0.2X SSC/0.1% Triton-X-100 at 56 ^{0}C for 20 min each. The samples were then cooled to the room temperature and washed twice for 10 min with TNTx (0.1 M Tris, 0.15 M NaCl, 0.3% Triton-X-100, pH 7.5 and filter-sterilized). Thereafter, the samples were blocked for 1.5 h in TNTx with 5% horse serum (Sigma-Aldrich, Cat. No. H0146) and 0.5% Western Blocking Reagent (Roche, Cat. No. 25263300). A peroxidase-coupled, anti-DIG-POD antibody (1:500 in blocking buffer, Roche, Cat. No. 11207733910) was incubated at 4 ◦C overnight. Next, the samples were washed with TNTx

(quick rinse, 5 min, 10 min and 6 times 20 min). Tyramide Signal Amplification (TSA) based development was performed in TSA buffer (2 M NaCl; 0.1 M Boric acid, pH 8.5; filter sterilized) with a FAM-conjugated tyramide (1:500 diluted), 1:1000 4IPBA (4-Iodophenylboronic acid, Sigma-Aldrich, Cat. No. 471933, 20 mg/ml stock in Dimethyl sulfoximide, DMSO) and 0.003% hydrogen peroxide.

FAM-conjugate was constructed as described by King and Newmark (2013). The samples were incubated for 15 min in the dark in the development mix, washed three times with PBST, post-fixated during 20 min in 4% formaldehyde/PBST, washed again thrice with PBST and then mounted with Immu-Mount (Thermo ScientificTM, 9,990,402). To determine the number of early progenitor cells/mm^2, the total number of fluorescent cells was normalised to the body size of the animals, which was determined using the ImageJ software (version 1.51, Na- tional Institutes of Health, Bethesda, MD, USA) by measuring the surface of the animals post fixation.

2.5.4 Comet assay

DNA damage was determined using an alkaline comet assay, based on Singh et al. (1988). A uniform cell suspension was obtained using a papain cell dissociation technique (Moritz et al., 2012). In brief, the worms (at least one animal per sample) were incubated in 2% L-cysteine HCl (with 5 M NaOH to pH 7.0) for 2 min at room temperature and washed with CMFH (25 mM $NaH_2PO_4 \cdot 2H_2O$, 142.8 mM NaCl, 102.1 mM KCl, 94.2 mM $NaHCO_3$, 0.1% BSA, 0.5% glucose, 15 mM Hepes, pH 7.2) before being cut into small pieces (in 250 ml CMFH). Papain solution (30 U/ml CMFH) with 0.2 M L-cysteine was added for dissociation (1 h at 26 ^{0}C), after which the pieces were disrupted by pipetting up and down. From then on, the samples were kept on ice to prevent damage. The samples were filtered with a 35 µm filter, washed with CMFH, and collected through centrifugation. After centrifugation, the supernatant was removed from each sample and the pellet was rewashed in CMFH and pelleted again (5 min, 350 rpm). Next the samples were resuspended in 1 ml CMFH, transferred to 1.5 ml tubes and pelleted once again. After this step, the supernatant was discarded, and the pellet was dissolved in 160 µl 0.8% low–melting- point agarose/PBS (preheated to 37 ^{0}C) by gently pipetting up and down. 70 µl of the sample (in duplicate, as a technical replicate on two separate slides) was immediately transferred to a 1% agarose coated slide, covered with a cover slip, and left on ice to solidify. Next the coverslips were removed, and the slides were immersed in lysis solution (2.5 M NaCl, 0.1 M Na_2EDTA, 10

mM Tris, 1% Triton X-100, 10% DMSO, pH 10.0) for 2–24 h at 4 ^{0}C (to remove cellular membranes and proteins form the DNA).

After washing twice with cold PBS, the slides were placed in an electrophoresis tank containing a cold alkaline solution (300 mM NaOH, 1 mM Na_2 EDTA, pH 13.0) for 20 min to allow the DNA to unwind. Electrophoresis was carried out at 0.95 V/cm over the platform and 300 mA for 10–20 min at 4 ^{0}C. Using a peristaltic pump, the electrophoresis buffer was recirculated from the anode to the cathode. Following elec- trophoresis, the slides were neutralised by a 5-min wash in cold PBS, followed by a 5-min wash in cold MilliQ water. SYBR Gold Nucleic Acid Gel Stain (1 μl/10 ml molecular water) DNA was used for staining (20 min in the dark) (Invitrogen, Cat. No. S11494) and image analysis was performed using a Zeiss fluorescence microscope equipped with the semiautomatic Metafer 4 image analysis system (Metasystems) using 10X objective magnification. Analysis was performed manually using Comet Assay IV software (Instem-Perceptive Instruments). An analysis of approximately 100 comets per sample (50 per technical duplicate) was performed and the relative amount of DNA damage was determined by measuring the tail moment of the average of two technical replicated slides. The tail moment is defined as the product of tail length and the proportion of the total DNA in the tail (Mozaffarieh et al., 2008; For- chhammer et al., 2010) and was calculated from 2 (technically) repli- cated slides.

2.6 Molecular responses

2.6.1. Gene expression analysis

For the gene expression analysis, RNA was isolated using a phenol-chloroform extraction procedure (Chomczynski et al., 2006) and was precipitated with Na-acetate and 70% ethanol. The nucleotide concentrations were determined on a Nanodrop ND-1000 spectrophotometer (NanoDrop® ND-1000, ISOGEN Life Science). All RNA samples were adjusted to the concentration of 200 ng. Genomic DNA was removed with the Turbo DNA free kit (Ambion® Thermo Fisher Scientific). cDNA was synthesised using Superscript TM III first-strand synthesis supermix (Thermo-fisher Scientific) according to the manufacturer's instructions. cDNA samples were stored at − 20 ^{0}C until their use for amplification by real-time qPCR.

Table 1. Nucleotide sequence and functions of specific primer pair of Housekeeping Genes (HKG) for flatworm *(Schmidtea mediterranea)* used in the study.

Gene	Function	5' – 3' Sequence
Cystatin	Inhibits peptidase families	F: AACTCCATGGCTAGAACCGAA R: CCGTCGGGTAATCCAAGTACA
gapdh	Glycolysis	F: GCAAAACATTATTCCGGCTTC R: GCACTGGAACTCTAAAGGCCA
rlp13	Ribosomal protein L13	F: AGGTGTCCCAGCTCCTTATGA R: GGCCCAATTGACAGAATTTC
beta - actin	Cytoskeleton protein	F: AGAACAGCTTCAGCCTCGTCA R: TGGAATAGTGCTTCTGGGCAT
gm2ap	Ganglioside catabolism, phospholipid metabolism, T – cell activation	F: CCGTCAGATTAAAGCTCGGTT R: TTTCGGACATTCGTTACCCAT
actin	Microfilament protein	F: AGAACAGCTTCAGCCTCGTCA R: TGGAATAGTGCTTCTGGGCAT

Table 2. Nucleotide sequence and functions of specific primer pair of genes of interest for flatworm *(Schmidtea mediterranea)* used in the study

Gene	Function	5' – 3' Sequence
cat	Oxidative Stress	F: CCATTTAGAAATTACGAAGTCGATG R: AAGTATCTTGGGTTATGTTGAGG
gr	Detoxification	F: GTAGTTGGCTTCCACGTTCTC R: GTTTCGTCGCTCCCATTCTC
gst	Detoxification	F: CACCAGAAGAGAAGAAAGAAGAAC R: TTTCTCCAGCGAAAAATCC
CuZn-sod	Oxidative stress	F: TTCATGCTGTATGCGTTTTG R: AACCGTGTTTACCAGGAGTTAGA
Mn-sod	Oxidative stress	F: TGGGCTTGGTTAGGGTTGAA R: AACGTCAAATCCTAGCAACGG
hsp70	Heat shock	F: GTAGCAATGAATCCCAACAA R: CAGTAACAACATCAAAGGACCAA
p53	Tumor suppressor	F: CCAATTATTTACCAAACCTCATCCT R: GGATCCCCAAAAACTGGAAC
bcl2	Apoptosis	F: GGGTCAGAGAAAATGGAGGA R: TATCCCCAGGGCCACTTT
bax	Apoptosis	F: CAAGTCGGCTTTTAATGATTTCTC R: AAACAGGTATACGATTGCGTTCCA
casp3	Apoptosis	F: ATTCAAGCCTGTCGTGGTG R: CAGCTTCAATTGGAATCTTTTCT
xpa	DNA repair	F: GAC CCG GAG CAG CAT AAG R: GAT TCG GCT GGC GTT TAT T
rad51	DNA repair	F: ATG TCA GAA TCC CGA TAC GC R: ATC AGC CAA CCG TAA CAA GG
cdc23	Cell cycle	F: AAAGGCGATTTAGCGGAAAG R; TCATTGCATTTGTGGTGAGG
cyclin-b	Cell cycle	F: GGTTTCCTACGCGAAAACAG R: CCGACCAATAGGATCAATGG
mt	Detoxification	F: CCATTACAGCCTTGTTTGTTG R: TTACCGGTGTAGTTGGTGCT

Real-time qPCR was performed in an optical 96-well plate (Applied Biosystems, Thermo Fisher Scientific, US) using Fast SYBR Green Master Mix (Applied Biosystems, Thermo Fisher Scientific, US) amplified and detected with the 7500 Fast Real-time PCR System (Applied Biosystems, Life Technologies, US) under universal cycling conditions. Primers efficiencies calculated as E = 10− 1/slope were evaluated by a four-point standard curve, prepared by a 1:3 serial dilution of cDNA. Efficiencies of 0.85–1.15 were accepted. Potential reference

genes were selected using the method by Rongying et al. (2007). The three most stable reference genes were determined by geNorm analysis. Gene expression analyses were performed according to the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (Table S1: see supplementary data) (Bustin et al., 2009). Gene expression (transcriptional) profiles of the genes related to the cellular redox state, cell cycle and (DNA) repair mechanisms were studied in all exposure setups. The relative quantification of each gene expression level was normalised according to the expression of the three reference genes. The relative gene expression was calculated using the 2− ΔCt method as described by Livak and Schmittgen (2001). All the primer sequences are listed in Tables 1 and 2.

2.6.2 Hydrogen peroxide quantification

The hydrogen peroxide (H_2O_2) content was determined spectro- photometrically using an OxiSelectTM hydrogen peroxide assay kit (Cell Biolabs, Inc., San Diego, US) according to the manufacturer's guidelines. Planarians were snap-frozen and homogenised in a 1x buffer. Homog- enates were centrifuged at 13,000 rpm for 1–2 min at 4 ^{0}C and the su- pernatant obtained was used for the H_2O_2 quantification. A small volume of the supernatant (10 µl) was diluted (1:10) and used for protein measurement. This was followed by preparing ADHP (10-Acetyl-3, 7-dihydroxyphenoxazine) and HRP (horseradish peroxidase) working solutions. The absorbance was measured at 544 nm in a FLUOstar Omega multi–mode microplate reader (BMG Labtech, Ortenberg, Ger- many) and the H_2O_2 concentration was calculated using a standard curve. The total protein content was measured separately using Bradford 1X dye reagent and with Bovine Serum Albumin (BSA) as standard.

2.6.3 Glutathione assay

For the measurement of glutathione, animals (10–12 animals/sample) were snap-frozen and stored at − 70 ^{0}C. Glutathione was measured by the recycling assay initially described by Tietze (1969) and adapted by Queval and Noctor (2007). Without pre-treatment of extracts, total glutathione, which is reduced glutathione (GSH) plus oxidised gluta- thione (GSSG), was measured at 412 nm. A specific measurement of GSSG was achieved by pretreatment of extract aliquots with 2-vinylpyridine (2-VP), as described by Griffith (1980). Frozen animals were dis- solved in 300 µl of 0.2 M HCl, equipped with glass beads of 2 µm diameter, and disrupted using a Retsch Mixer Mill MM 200 (Retsch). Then, 280 µl of the homogenate was transferred to a new tube. This was neutralised first with 30 µl of 0.2 M $NaH2PO_4$ and adjusted to a pH of 5.6 with approximately 230 µl of 0.2 M NaOH. Since the addition of NaOH

leads to precipitation of the mucus, the supernatant was transferred to a fresh tube after centrifugation. Next, 340 µl of the sample was trans- ferred to new tubes for the assessment of GSSG while the remaining 180 µl was used for the total GSH assay. To measure total GSH, quadruple aliquots of 40 µl of the neutralised extract were placed in a 96-well plate (Greiner Bio-One, Kremsmünster, Austria). The total reaction volume of 200 µl also contained 0.1 M NaH_2PO_4 (pH 7.5), 5 mM EDTA, 0.5 mM NADPH, 0.6 mM DTNB. Again, the reaction was initiated by the adding 0.2 U of GR to all but one sample (no-GR control). To measure GSSG, samples were first treated with 1 µl of 2-VP for 30 min at RT to complex GSH. To remove excess 2-VP, the derivatised solution was centrifuged twice. Quadruple aliquots of 80 µl neutralised extract were added to 96-plate wells. The total reaction volume of 200 µl also contained 0.1 M NaH_2PO_4 (pH 7.5), 5 mM EDTA, 0.5 mM NADPH, 0.6 mM DTNB. Again, the reaction was initiated by the addition of 0.2 U of GR to all samples but one (no-GR control). After mixing the reaction mixture on a shaker, the increase of absorbance (GR-dependent reduction of DTNB) was monitored for 15 min with the Fluostar Omega multi-mode microplate reader (BMG Labtech, Ortenberg, Germany). Standards were run simultaneously in the same plates as duplicate assays from 0 to 2 nmol GSH and from 0 to 0.4 nmol GSSG respectively. Oxidised glutathione standards were also treated with VPD. The results were normalised to the size of the worms.

2.7 Statistical analysis

The statistical analysis was performed using the Prism 8 statistical software (GraphPad, San Diego, US). In all the experiments, each parameter was analysed separately using appropriate controls. All the experimental results were analysed by a non-parametric test (Kruskal – Wallis and post-hoc comparision test) based on ranking. A p-value less than 0.05 was considered statistically significant.

3. Results

In this study, we aimed to determine the combined toxicity of Cu and Cd in both regenerating and adult planarians *(Schmidtea mediterranea).* Regenerating organisms were used as a proxy to study developmental effects, as developing organisms are often not included in current risk assessments. Metal uptake and toxicodynamic responses were assessed after 7 and/or 14 days of exposure to Cu and Cd alone, and as a binary mixture.

3.1 Metal accumulation

Cu and Cd concentrations were determined in both regenerating and adult animals (Fig. 1A and B, Table S2, S3: see supplementary data). In regenerating animals, single and mixed exposure scenarios resulted in a comparable Cu accumulation in both head and tail fragments ($p < 0.05$). Cd concentrations also increased significantly in head and tail fragments under all Cd exposure conditions ($p < 0.05$), but the increase was higher when both Cd and Cu were applied together. In adult animals, Cu concentrations were only significantly increased in the single exposure set-up of the 7 days exposure group ($p < 0.05$). Exposure to Cd led to increased Cd levels ($p < 0.05$) at both time points (7 and 14 days) under all exposure conditions.

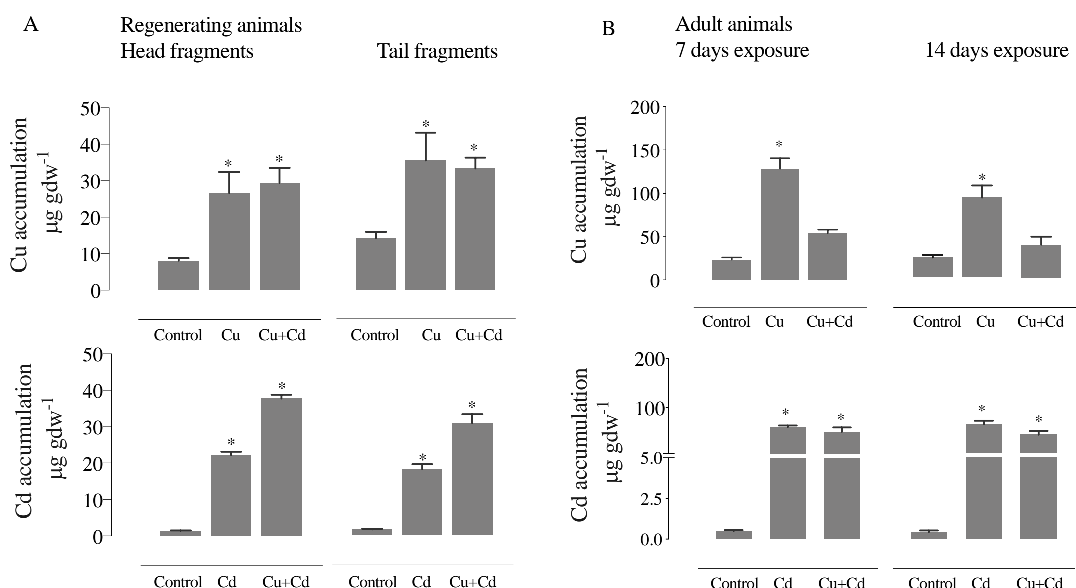

Fig. 1. Metal accumulation in regenerating and adult planarians *(Schmidtea mediterranea).* Graphs representing Cu and Cd concentrations in (A) regenerating and (B) adult (intact) animals, exposed to 20 μM Cu, 10 μM Cd and a combination of 20 μM Cu and 10 μM Cd compared to control (unexposed) animals. The values indicated in the figures are averages ± standard error of mean (SEM) of minimum 6 biological replicates per condition. A non-parametric test (Kruskal – Wallis test and post hoc comparison test), based on ranking was used to compare the treatment and control groups. *$p<0.05$. dw = dry weight

3.2 Morphology, growth, and survival

In both regenerating and adult animals exposed to the Cu and Cd mixture, a decline in survival was observed. This decline occurred earlier in regenerating animals (at 7 days exposure; head: 10%, 02/20, tail: 10%, 02/20, at 14 days exposure; head: 60%, 12/20; tail 60%, 12/ 20), as compared to the adults (at 14 days exposure - 40%) (Fig. 2). A 15% decline

(03/20) in survival was observed in the regenerating animals exposed to Cu alone (Figs. 2 and Fig. 3).

Morphological analyses of regenerating and adult animals were carried out to determine the effect of all treatments on morphology and growth. The animals exposed to the metal mixture gradually developed morphological aberrancies, such as tissue lesions (loss of epithelial integrity), contracted body (reduced body surface area), head regression (lyses or distortion of anterior structure) and outgrowths (tumorigenic malformations). As shown in Figs. 2A, 3A and 50% of the regenerating (09/18) and adult (10/20) animals that were alive, showed an abnormal morphology after 7 days of exposure. After 14 days of exposure, this was 75% (06/08) in head fragments, 87% (07/08) in tail fragments and 66% (08/12) in adult animals exposed to both Cu and Cd simultaneously. Similar but milder aberrancies (15%; 03/20) were observed in regenerating animals exposed to Cu alone after 7 days of exposure, whereas no visible alterations were observed in the Cu-exposed adults, or in the Cd- exposed animals. The regenerative capacity was evaluated by measuring the blastema size relative to the total body area. After 14 days of exposure, total body sizes of all metal-exposed regenerating animals were smaller, but only significantly reduced in the combined exposure groups ($p < 0.0001$) (Figs. 2B and 3B). Head and tail fragments were respectively 67% and 70% smaller ($p < 0.0001$) compared the unexposed animals.

Adult animals exposed to a combination of Cd and Cu were 56% smaller ($p < 0.05$) than the unexposed animals, and this after an exposure period of 14 days (Fig. 3). The blastema formation in regenerating animals was significantly affected by the combined expoure. Both head and tail fragments showed a significant decrease in blastema growth ($p < 0.001$) after 7 days of exposure to the mixture (Fig. 2).

To estimate the impact of metal exposures on developing organisms, all regenerating animals were put in a metal-free medium (cultivation medium) after exposure, whereafter the recovery potential was monitored (Fig. S4: see supplementary data). Aberrancies were still present in the tail fragments exposed to Cd and Cu together (30%, 06/20), even at the end of the recovery period. The blastema sizes of the animals exposed to the mixture were significantly lower during continuous exposure as well as after a recovery period ($p < 0.0001$).

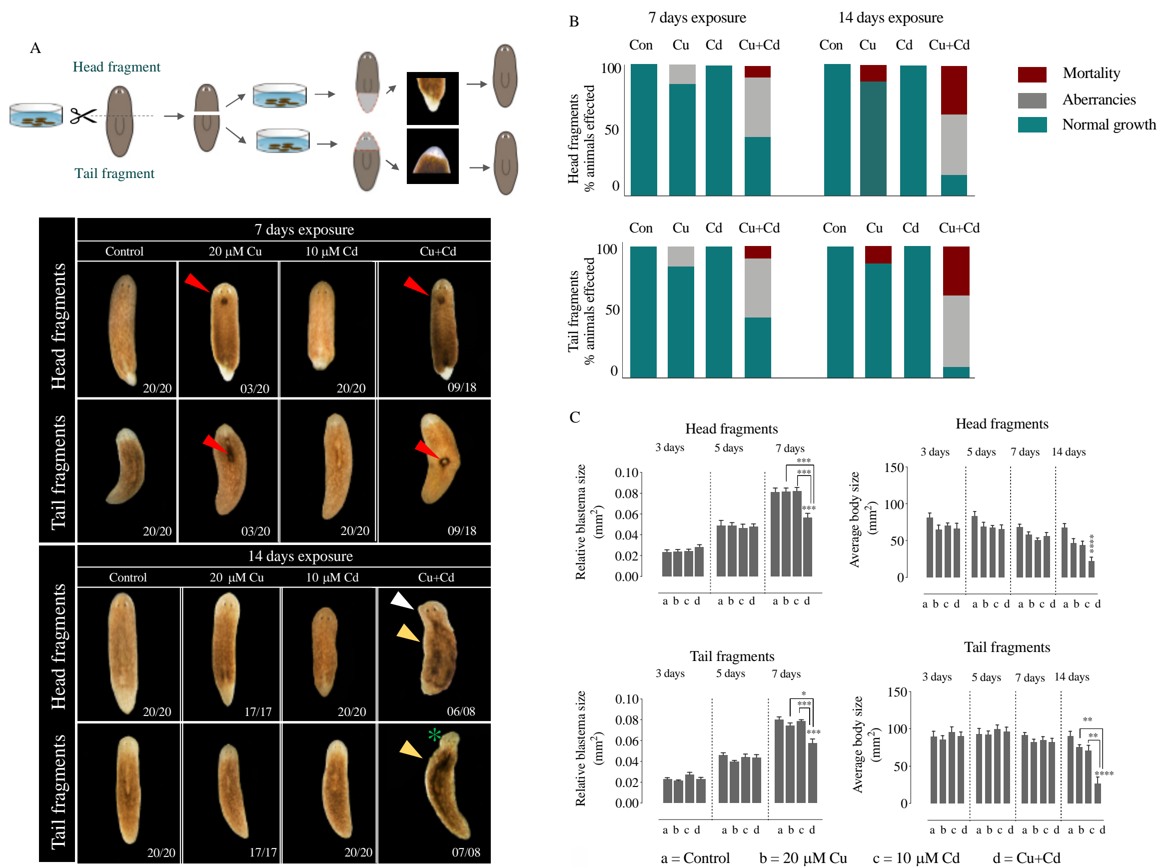

Fig. 2. Morphological screening in regenerating planarians (*Schmidtea mediterranea). (*A) Representative images of the observed morphological changes in regenerating animals, exposed to different exposure conditions as indicated. The most frequently observed aberrancies were lesions (red arrow). Exposure to Cu + Cd caused body contraction and/or curvature (yellow arrow), formation of outgrowths (white arrow) and head regression (green asterisk). The morphology of all metal- exposed animals was compared to control (unexposed) animals. After 14 days, unexposed animals were fully regenerated, whereas regeneration failed in Cu + Cd exposed animals. (B) Quantification of growth and survival after 7- and 14-day exposure to 20 µM Cu, 10 µM Cd and a combination of 20 µM Cu and 10 µM Cd compared to control animals. Normal growth refers to a normal increase in animal size; aberrancies mean visible malformations, such as, body lesions, body contraction and/or curvature, outgrowths, and head regression; mortality refers to death. (C) Quantification of blastema size (unpigmented newly formed tissue, expressed relative to the total body area and whole-body size in regenerating head and tail fragments. Values indicated in the figure are the averages ± standard error of mean (SEM) of 20 biological replicates of 2 independent experiments. A non-parametric test (Kruskal – Wallis and post-hoc comparison test), based on ranking was used to compare the treatment and control groups. **p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001*. Scale bar = 1000 µm.

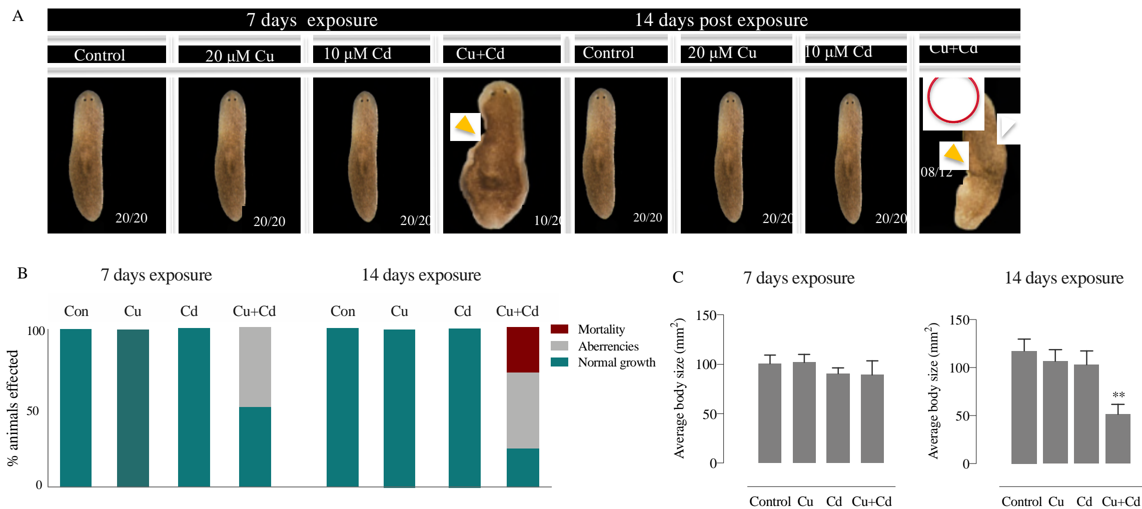

Fig. 3. Morphological screening in adult planarians (Schmidtea mediterranea). (A) Representative images of the observed morphological changes in adult animals, exposed to different exposure conditions as indicated. Exposure to the Cu + Cd mixture caused severe aberrancies such as body contraction and/or curvature (yellow arrow) and outgrowths (white arrow) as well as head regression (red circle). (B) Quantification of growth and survival after 7- and 14-day exposure to different exposure conditions. Normal growth refers to a normal increase in animal size; aberrancies mean visible malformations, such as, tissue lesions, body contraction and/or curvature, outgrowths, and head regression; mortality refers to death. (C) Quantification of whole-body size 7 and 14 days after exposure to 20 µM Cu, 10 µM Cd and a combination of 20 µM Cu and 10 µM Cd. Values indicated in the figure are the averages ± SEM of 20 biological replicates of 2 independent experiments. A non-parametric test (Kruskal – Wallis and post-hoc comparison test) based on ranking was used to compare the treatment and control groups. ***$p < 0.01$*. Scale bar = 1000 µm.

3.3 Neurophysiology and neurodevelopment

We tested the locomotor activity (LMA, distance covered per unit of time) under both the single and mixed exposure conditions of regenerating animals, 7- and 14-days post amputation (Fig. 4). Untreated animals showed a normal LMA throughout the duration of the experiment, whereas the animals exposed to metal mixtures showed a reduced LMA ($p < 0.0001$). They also developed distinct behavioural patterns such as caterpillar-like movement and contractions. Cu-exposed head fragments displayed a temporary decrease ($p < 0.05$) in LMA, whereas the LMA of Cd-exposed animals gradually decreased over time. To assess whether these behavioural effects are related to neural development, the nervous system was visualized to study the regeneration of the cephalic brain ganglia (in tail fragments). Compared to unexposed animals, which developed normally, a significant decrease in the ratio of brain ganglia width to head area ($p < 0.01$) was observed after a combined exposure to Cu and Cd (Fig. 4). To further verify the impact of metal exposures on

neuroregeneraion, the animals were transferred to a metal-free freshwater medium, and the recovery was monitored. An incomplete development of the brain ganglia was also observed in the animals exposed to the mixture in a recovery situation (83%, 05/06) (*p < 0.01*) (Fig. S4).

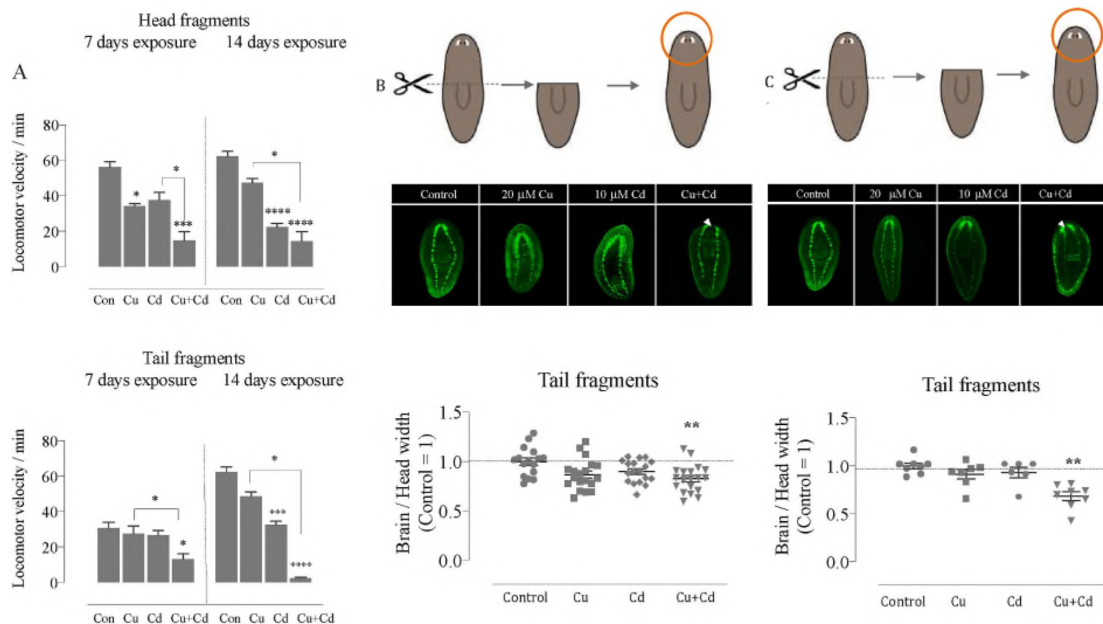

Fig. 4. Neuroregeneration in planarians *(Schmidtea mediterranea).* (A) Locomotor activity in regenerating head and tail fragments exposed to 20 µM Cu, 10 µM Cd and a combination of 20 µM Cu and 10 µM Cd compared to control (unexposed) animals. (B) Neuroregeneration in control and exposed animals 7- and 14-days post amputation in different experimental conditions. White arrows represent defect in neuroregeneraion (not fully developed ganglia). (C) Quantification of brain sizes of different treatment groups, after recovery in plain medium, 7 days post amputation. Relative sizes of brain ganglia are calculated as a ratio of the brain width to head width. The values indicated in the figure are the averages ± SEM of ≥6 biological replicates of 2 independent experiments. A non-parametric test (Kruskal – Wallis and post-hoc comparison test) based on ranking was used to compare the treatment and control groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Scale bar = 500 µm.

3.4 Cell cycle responses

After 7 days of single and mixed exposures, cell cycle responses were assessed by examining the *in vivo* mitotic activity of stem cells, the amount of apoptotic and the amount of early progenitor cells (NB.21.11e). In both regenerating and adult animals, a combined exposure of Cu and Cd induced a significant increase in the amount of stem cell division (heads: $p < 0.05$, tails: $p < 0.01$, adults: $p < 0.0001$) (Fig. 5A and B). In adults, the number of dividing cells was also significantly increased after exposure to Cd alone ($p < 0.01$). An increase in apoptosis was only observed in adult animals exposed to the mixture whereas Cd exposure

resulted a decrease of apoptotic cells in regenerating head fragments (Fig. 5C and D) (*$p < 0.05$*). The amount of early progenitor cells was not altered in any of the exposure conditions (Fig. 5E).

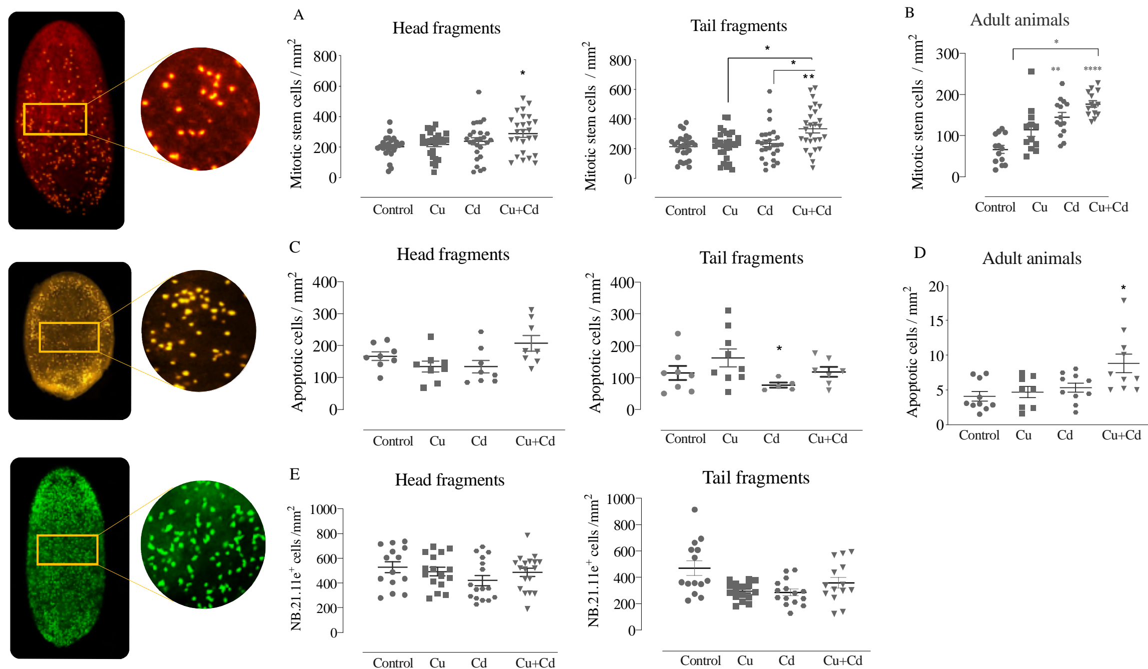

Fig. 5. Cell cycle responses in regenerating and adult planarians *(Schmidtea mediterranea).* Quantification of stem cell proliferation by whole mount immunostaining with anti–H3P antibody in (A) regenerating and (B) adult (intact) animals exposed to 20 µM Cu, 10 µM Cd and a combination of 20 µM Cu and 10 µM Cd compared to control (unexposed) animals. The number of stem cells was normalised to the body area. The values indicated in the figure represent the average ± SEM of ≥ 10 biological replicates of 2 independent experiments. (C) quantification of apoptosis in response to metal exposures (20 µM Cu, 10 µM Cd and a combination of 20 µM Cu and 10 µM Cd) compared to control animals The number of apoptotic cells was normalized to the total body area of the worms. The values indicated in the figure are averages ± SEM of ≥ 6 animals per condition of 2 independent experiments. (C) quantification of stem cell differentiation NB.21.11e (early progenitor cells) in regenerating animals (head and tail fragments) exposed to 20 µM Cu, 10 µM Cd and a combination of 20 µM Cu and 10 µM Cd compared to control animals. Minimum 8 biological replicates were used per condition of 2 independent experiments. The number of differentiating cells was normalized to the total body area. In all experiments, a non-parametric test (Kruskal – Wallis test) based on ranking was used to compare the treatment and control groups. **$p<0.05$, ** $p<0.01$, **** $p<0.0001$*. Scale bar = 100 µm.

3.5 Molecular responses

The transcriptional profiles of the genes related to the cellular redox state, cell cycle and (DNA) repair mechanisms were studied in all exposure setups (Fig. 6A). In regenerating animals, the transcription of the antioxidative genes glutathione reductase *(gr:* $p < 0.01$),

glutathione transferase (*gst)* (head: $p < 0.01$, tail: $p < 0.05$) and metallothionein (*mt)* (head: $p < 0.05$, tail: $p < 0.01$) was upregulated in both head and tail fragments exposed to the metal mixture. Similar responses were observed in the Cd-exposed head fragments ($p < 0.05$), whereas only *gr* was upregulated during Cu exposure (head: $p < 0.05$). Cd-exposed and Cu + Cd-exposed head fragments also showed an upregulation of the cell cycle checkpoint protein *cdc23* ($p < 0.05$). Apoptosis-related genes *blc2 (p < 0.001,) bax* ($p < 0.05$) *and casp3* (p < 0.05) were upregulated in the tail fragments of the combined exposure group, an upregulation that was also observed during Cd exposure *(bcl2: p < 0.05*).

To validate the transcriptional findings in regenerating animals, we quantified the hydrogen peroxide (H_2O_2) levels and glutathione content. Our results revealed a similar increase in H_2O_2 levels in the Cu- and combined exposure groups ($p < 0.05$), whereas no significant increase was observed in the animals exposed to Cd (Fig. 6B). A decrease in total glutathione content, and a corresponding increase in the GSSG:GSH ratio was observed in the head fragments exposed to the mixture ($p < 0.05$). Tail fragments showed a similar, but non-significant, trend in total glutathione content (Fig. 6C, D).

In adult animals, increased mRNA levels of *gr* (Cu: $p < 0.05$, Cu + Cd: 0.01), *p53* ($p < 0.05$), *bax* (Cu: $p < 0.01$, Cu + Cd: $p < 0.05$), *casp3* (Cu: 0.05, Cu + Cd: $p < 0.01$) and *rad51* (Cu: p < 0.01, Cu + Cd: $p < 0.05$) were observed for both Cu- and Cu + Cd-exposed animals after an exposure of 7 days. In addition, Cu + Cd-exposed animals showed an upregulation of *cdc23* ($p < 0.05$) and *mt* ($p < 0.05$). After 14 days of exposure, the expression of *gst* ($p < 0.01$), *hsp70* ($p < 0.05$), *xpa* ($p < 0.05$) genes was significantly increased in the animals exposed to the metal mixture (Fig. 7A). To validate the transcriptional findings in adult animals, we quantified DNA damage by the alkaline comet assay. DNA damage, indicated by enhanced tail moments (tail length x % DNA in tail) were observed in all the exposure groups, but were significant in the Cd- ($p < 0.05$) and Cu + Cd-exposed animals ($p < 0.001$). The effect was stronger in the animals exposed to the mixture (Fig. 7B).

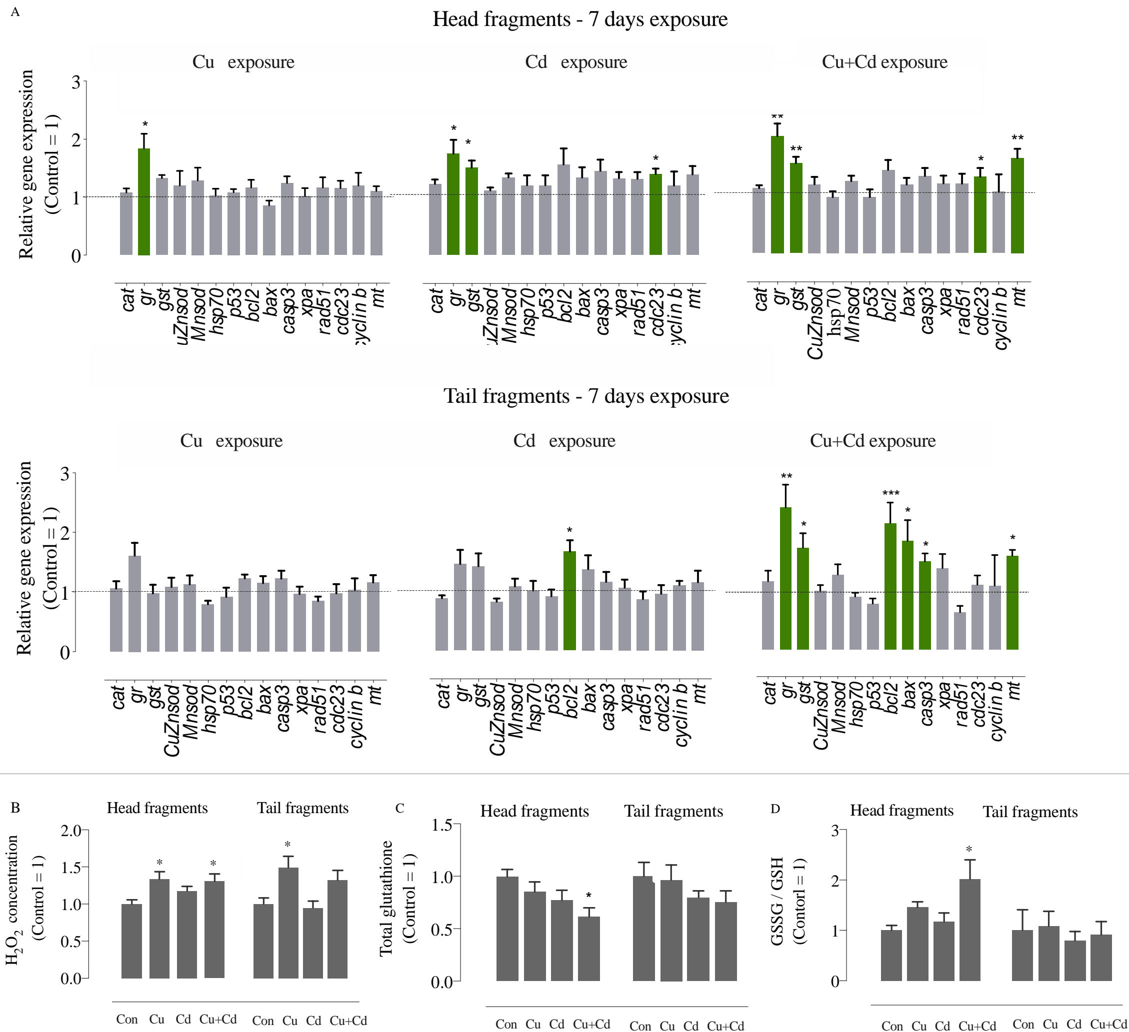

Fig. 6. Molecular responses in regenerating planarians (*Schmidtea mediterranea)*. Transcript level of genes related to antioxidative, apoptosis, cell – proliferation, DNA repair related genes, after 7 days exposure in regenerating head and tail fragments. *A*nimals were exposed to 20 µM Cu, 10 µM Cd and a combination of 20 µM Cu and 10 µM Cd. The values indicated in figure are averages ± SEM of minimum 6 biological replicates (B) hydrogen peroxide (H_2O_2) in regenerating head and tail fragments after 7 days exposure in different exposure conditions (20 µM Cu, 10 µM Cd and a combination of 20 µM Cu and 10 µM Cd). The values indicated in figure are the averages ± SEM of ≥ 6 biological (pooled data of ≥ 2 independent experiments). (C) total glutathione and (D) GSSG / GSH ratio in different exposure scenarios (20 µM Cu, 10 µM Cd and a combination of 20 µM Cu and 10 µM Cd). The values indicated are averages ± SEM of minimum 10 biological replicates of 2 independent experiments. In all experiments a non-parametric test (Kruskal – Wallis test), based on ranking was used to compare the treatment and control groups. based on ranking was used to compare the treatment and control groups **p<0.05, **p<0.01*, ****p<0.001.*

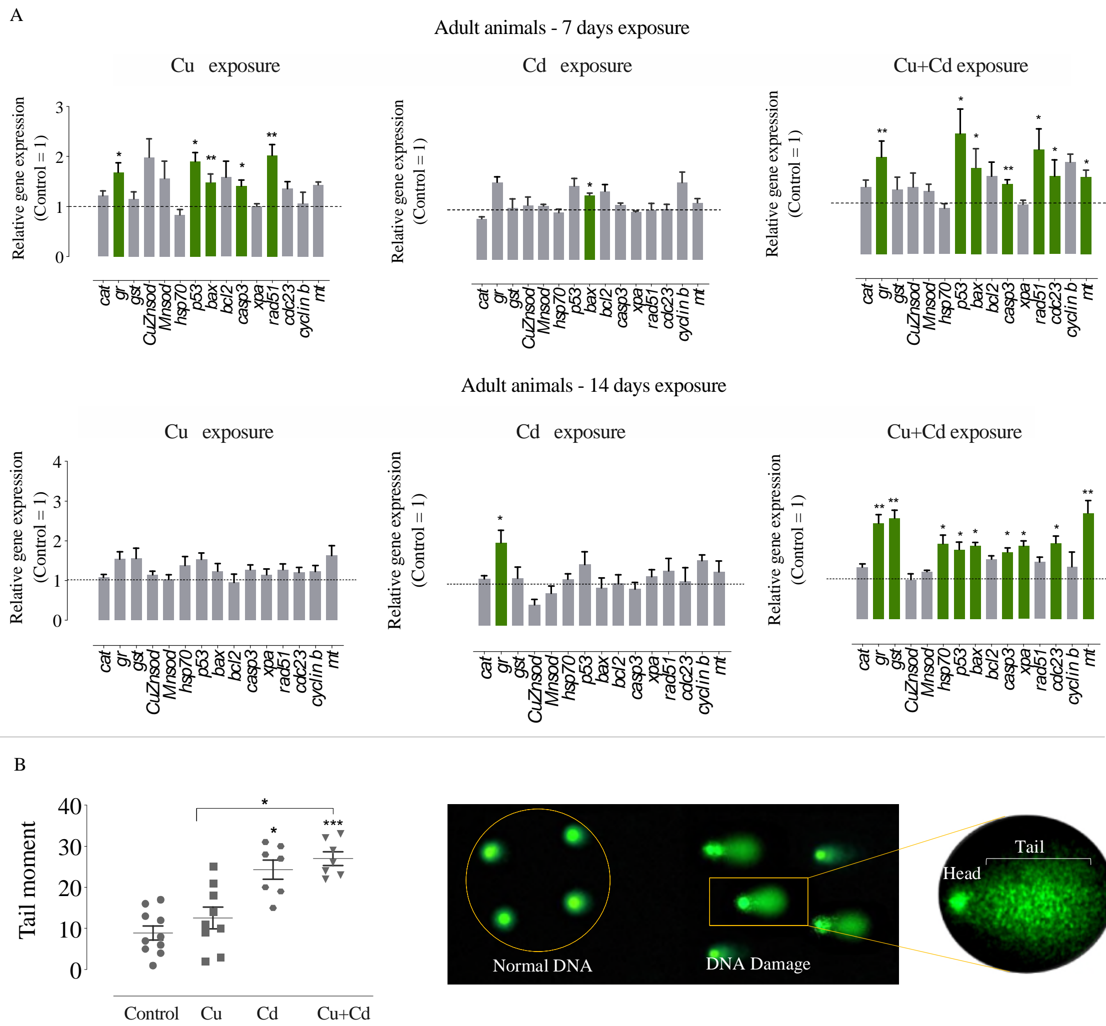

Fig. 7. Molecular responses in adult planarians (*Schmidtea mediterranea*). (A) Transcript level of genes related to antioxidative, apoptosis, cell – proliferation, DNA repair related genes, after 7 days exposure in adult animals. Animals were exposed to 20 µM Cu, 10 µM Cd and a combination of 20 µM Cu and 10 µM Cd. The values indicated in figure are averages ± SEM of minimum 6 biological replicates. (B) DNA damage (tail moment), 7 days post exposure to 20 µM Cu, 10 µM Cd and a combination of 20 µM Cu and 10 µM Cd. DNA damage is expressed as tail moment (tail length x % DNA in tail). The values indicated in the figure are averages ± SEM of ≥ 6 animals per condition of 2 independent experiments. In all experiments, a non-parametric test (Kruskal – Wallis test), based on ranking was used to compare the treatment and control groups. **p<0.05, **p<0.01, ***p<0.001, ***p<0.0001*. Scale bar = 100 µm.

4. Discussion

Synergistic interactions of metals are a major issue. Metal mixtures induce effects at different thresholds, and scientific evidence is needed to underpin regulatory decisions (Wah Chu and Chow, 2002; Barbee et al., 2014; Crémazy et al., 2018; Moyson et al., 2018,

2019; Pilehvar et al., 2019). Especially for the early life stages of developing organisms, mechanistic information is lacking. In the current study, regenerating and adult planarians (*Schmidtea mediterranea)* were used to investigate the *in vivo* toxicity of single and combine exposures of 20 µM Cu and 10 µM Cd during 7 days and/or 14 days. *Schmidtea mediterranea,* an established invertebrate model organism for stem cell and regeneration research, was used to assess developmental effects, using its regenera- tion capacity as a proxy for tissue development.

An exposure to Cu and Cd led to their accumulation in all the treated animals. Metal interactions and their toxicokinetic profiles, however, altered during different developmental stages. The metal-binding protein metallothionein was upregulated in al mixed exposure scenarios, but the simultaneous presence of Cd caused a decrease in Cu accumulation in adult animals, indicating an Cd-induced inhibitory effect on Cu uptake. In regenerating animals, the presence of Cu resulted in an increased Cd accumulation. In literature, different inhibitory trends have been described, both in favour of Cd and Cu uptake. Komjarova and Blust (2008) found an inhibition of Cu uptake by Cd in *Daphnia magna*, whereas the opposite was found in *Danio rerio* (Komjarova and Blust, 2009a; Komjarova and Bury, 2014). We hypothesize that the observed inhibitory effect of Cd on Cu uptake in adult animals is most likely due to its preferential uptake or out competitive action at transporters such as calcium channels (ECAC), and a subsequent rerouting of cadmium via divalent metal ion transport protein (DMT1) as suggested by Komjarova and Bury (2014) and Pilehvar et al. (2019). Although total metal accumulation was significantly higher in adults, it caused less severe effects then in regenerating animals. Also, for the single Cu exposure, a higher in- ternal Cu load did not induce morphological effects in adults, whereas it did in regenerating animals. These findings indicate that the extent of metal accumulation is not always a reliable predictor of toxicity, especially not in a setting where co-exposure occurs. Moreover, the different uptake-effect ratios show that is always important to include both early and adult life stages in risk assessment or toxicity screens.

Differences in sensitivity were visible at all biological levels. Lethality mainly occurred in the animals exposed to Cd and Cu simultaneously (head 60%, tail 60%; adults 40%) (Figs. 2. Fig. 3) and appeared earlier in regenerating animals. Behavioural effects were also more prominent in the animals exposed to the combined exposure and included a decreased locomotor activity (Fig. 3) and distinct behavioural patterns such as caterpillar like

movement and contractions (Raffa and Rawls, 2008; Hagstrom et al., 2016). Stronger effects of metal mixtures, both at morphological and physiological levels, were also observed in other organisms such as rats, snails, fish and roundworms (Rai et al., 2010; Barbee et al., 2014; Crémazy et al., 2018; Moyson et al., 2018, 2019). In single exposure scenarios, 15% of the Cu-exposed regenerating animals died, albeit at a later time point and after severe morphological damage (at 14 days of exposure). The higher sensitivity towards Cu can be attributed to cellular detoxification systems that regulate intracellular metal levels by detoxifying and excreting metals and indicates that additional parameters are needed to assess the type of toxicity and concurrent defence strategy. (Traudt et al., 2016, 2017).

Toxicity starts long before lethality, and detailed information is needed to predict adverse effects on growth and behaviour (Anderson et al., 2001; Raffa and Valdez, 2001; Jiang et al., 2016). We used the ability of planarians to synchronize and modulate their body size according to environmental changes (Oviedo and Alvarado, 2003), to compare the effects of single and combined exposures on regeneration and growth. Morphological aberrations appeared in both regenerating and adult animals after exposure to Cd and Cu simultaneously (Figs. 2 and 3). The more pronounced morphological alterations in response to the mixture were tissue lesions, contracted body, head regression and outgrowths. Similar but milder effects were observed in Cu-exposed animals, whereas no such effects were observed in animals exposed to Cd alone. A combined exposure to Cd and Cu also interfered more strongly with the regeneration capacity, compared to the single metal exposures (Figs. 2 and 3). When animals were exposed to metal mixtures prior to the induction of regeneration, their average blastema size was significantly smaller in all the experimental setups, regardless of if exposure was stopped after amputation. Apart from the increased sensitivity of regenerating animals, our results show that the sensitivity between regenerating head and tail fragments also varies. As such, outgrowths were mainly seen in head fragments.

Underlying the behavioural changes and regenerative defects, we observed that the animals were unable to regenerate their nervous system. Planarians are unique in the animal kingdom as they are able to regenerate their brain, including a fully functional nervous system (Cebriá, 2007; Brown and Pearson, 2017). A significant decrease in cephalic brain formation was observed after a combined exposure to the Cu and Cd mixture, which coincides with previous studies that described the neurotoxicity of Cu and Cd in planarians

separately (Knakievicz and Ferreira, 2008; Wu et al., 2014). This probably resulted in the observed behavioural effects, although we cannot rule out direct effects on muscle contraction.

To further understand the underlying mechanisms of toxicity, we analysed cellular and subcellular processes related to cell cycle dynamics in all metal-exposed planarians. A combined exposure to Cu and Cd caused a significant increase in stem cell proliferation, in both regenerating and adult animals, which was also observed in Cd-exposed adults (Fig. 5A and B). Cell proliferation can be considered as one of the first events in tissue remodelling and repair, during development as well as during homeostasis (Biteau et al., 2011; Matson and Cook, 2017). However, over-proliferation may occur and result in the accumulation of an abnormal number of cells resulting in pathological conditions including cancer(Zhang etal.,2011;Gérard and Goldbeter,2014; Wiman and Zhivotovsky, 2017). We observed tissue outgrowths in regenerating head fragments and adults. To further validate the effects on cell proliferation, we analysed the transcriptional activity of different cell cycle related genes in all metal-exposed animals. The activity of cell division cycle protein *cdc23,* was transcriptionally upregulated in regenerating head fragments and adult animals. In other organisms, *cdc23* is a critical regulator of cell proliferation and cell cycle progression and its role in cancer biology is well documented (Zhang et al., 2011). Along with cell cycle genes, we determined whether the activity of *p53* and other apoptotic genes was transcriptionally altered in both regenerating and adult animals (Figs. 6A and 7A). In planarians, *p5*3 has a broader function than its known role as tumour suppressor (Van Roten et al., 2018). It has been shown to participate in tumour suppression and in modulating stem cell self-renewal in planarians (Pearson and Sanchez Alvarado, 2010). Adult animals showed an upregulation of *p53* as well as *bax* and *casp3,* which play a key role in the apoptotic pathway (Pawlowski and Kraft, 2000; Zamorano et al., 2012; Brentnall et al., 2013; Wang et al., 2016; Beroske et al., 2021). Regenerating tail fragments, like adult animals, also showed an upregulation of *p53, bax and casp3*. We assume that the enhanced stem cell proliferation in the ani- mals exposed to Cu and Cd simultaneously is to compensate for the cell loss through apoptosis (Fan and Bergmann, 2008; Portt et al., 2011; Diwanji and Bergmann, 2019). Apoptosis was not affected in regenerating animals, except for a decrease in the Cd-exposed regenerating head fragments (Fig. 5C and D), which could be due to the ability of Cd to inhibit programmed cell death (Filipič, 2012). Overall, both molecular and cellular profiles again

indicate that the toxicity mechanisms vary between regenerating head and tail fragments, and that regional factors modulate those differences in sensitivity.

The redox balance is known to be different in different body regions (Jaenen et al., 2021; Bijnens et al., 2021). Moreover, oxidative stress, an imbalance between reactive oxygen species (ROS) and antioxidants, is often put forward as a fundamental molecular mechanism underlying metal-induced toxicity (Valko et al., 2005; Zhang et al., 2014; Morcillo et al., 2016; Chen et al., 2018, Ferrer et al., 2010; Sies and Jones, 2020). Under carefully controlled conditions, ROS have an important role as signaling agents but cause cellular dysfunctions when produced in excessive quantities, due to their ability to oxidize all major bio- molecules including lipids, proteins and nucleic acids (DNA and RNA) (Ercal et al., 2001; Butterfield et al., 2010; Arimon et al., 2015). There is accumulating evidence implicating oxidative stress mediated neuro- toxicity both in humans and animals as a result of Cu and/or Cd intoxication (Rotilio et al., 2000; Rossi et al., 2006; Yang et al., 2007; Wang and Du, 2013; Jiang et al., 2014; Branca et al., 2020). To check whether a disturbed redox balance underlies the observed morphological, developmental, and neurophysiological alterations, we assessed the transcriptional patterns of different categories of antioxidative in both regenerating and adult animals (Figs. 6A and 7A). In regenerating animals, the transcription of antioxidative genes (*gr, gst*) was upregulated in the animals exposed to the metal mixture. *Gr* and *gst* are key antioxidant defense enzymes involved in the glutathione metabolism (Jozefczak et al., 2012; Kısa, 2017). Their upregulation was also observed in regenerating head fragments after a single exposure to Cu and Cd. To further validate these redox alterations at the cellular level, we measured hydrogen peroxide (H_2O_2) and glutathione (GSH) in regenerating animals. H_2O_2 is a reactive oxygen species that is involved in redox sensing, signaling and regulation (Sies, 2017), and is kept in balance by GSH, among others (Pompella et al., 2003). H_2O_2 levels were elevated both in Cu- and Cu + Cd-exposed animals. Alterations in the glutathione content were observed solely in the head fragments of the animals that were exposed to the metal mixture. A consistent upregulation of antioxidative gene *gr* was also observed (7 and 14 days exposure) in the Cu + Cd-exposed adult animals, which additionally led to the upregulation of *gst* after an exposure of 14 days. Exposure to Cu also led to an upregulation of *gr,* but this was only temporarily. Cd exposure caused an upregulation of *gr* at later time point (14 days exposure). We assume that increases in glutathione content counteracted the induced oxidative stress or free metals, as GSH chelates the metals and subsequently sequester the ligand–metal complexes (Cobbett, 2000; Jozefczak et al., 2012;

Presnell et al., 2013). Metal sequestration also occurs via metallothioneins *(mt),* which in our study was transcriptionally increased under mixed exposure.

As can be seen from the gene expression results, redox profiles vary between regenerating and adult animals. Despite less severe morpho- logical effects, more genes were significantly upregulated in adults, probably to counteract the induced damage, or because they were already activated during developmental processes in regenerating ani- mals. Similar patterns were observed in the upregulation of DNA repair genes such as *rad51* and *xpa* in adults. *Rad51* was transcriptionally upregulated after 7 days of exposure and *xpa* at later time point (14 days of exposure). Exposure to Cu also resulted in the upregulation of *rad51*, whereas no transcriptional response to DNA damage was detected in Cd- exposed animals. *Rad* proteins are crucial factors in DNA repair and are known to function early in response to damaged DNA to arrest the cell cycle progression (Lindsey-Boltz et al., 2001; Gachechiladze et al., 2017). *Xpa* proteins play important role as a scaffold that coordinates damaged DNA and other proteins to ensure adequate excision of DNA lesions (Sugitani et al., 2016). Increased levels of DNA damage were confirmed via the comet assay during Cd and mixed exposure. We cannot rule out to what extent the observed DNA damage is related to the increased apoptosis, since these animals also showed an upregulation of the apoptotic genes *bax* and *casp3* at both time points (7 and 14 days exposure). In general, we attribute the variation in sensitivity be- tween regenerating and adult animals to the differences in the repair capacity, allowing accumulation of DNA damage in adults. DNA repair pathways are known to become less efficient or somewhat deregulated with age leading to accumulation of DNA damage (Gorbunova et al., 2007; Moskalev et al., 2013; Lagunas-Rangel and Bermúdez-Cruz, 2019).

Conclusion

This study was set out to determine the effects and underlying mechanisms of single and mixture exposure to Cu and Cd in regenerating and adult planarians *(S. mediterranea).* Our results show impairments in tissue homeostasis, growth, and development as a result of the combined toxicity of Cu and Cd. One of the more important findings of this study is an increased sensitivity of developing tissues and potentially different mechanisms of toxicity operating in the two distinct life stages, probably due to differences in the repair capacity. In both regenerating and adult animals, oxidative stress was a major pathway underlying metal toxicity, which in adults led to DNA damage. Additionally, the evidence from this study

suggests that metal mixtures can cause irreparable damage to developing organisms, but that the level of metal accumulation is not necessarily a reliable indicator of toxicity.

Supplementary Data

Supplementary table S1. MIQE guidelines concerning qPCR experiment.	
Experimental design	
Definition of experimental and control groups	Experimental groups - Regenerating planarians: (head and tail fragments) 3, 5, 7 and 14 days exposed to 20 µM Cu, 10 µM Cd singly and as a mixture - Homeostatic planarians: 7 and 14 days exposure to 20 µM Cu, 10 µM Cd singly and as a mixture - Control groups: planarians in culture medium
Number within each group	n ≥ 6
Sample	
Procedure and / or instrumentation	Frozen animals were disrupted by chemical lysis in 200µl RNA lysis/binding buffer (Qiagen, Venlo, the Netherlands). RNA was isolated using a phenol-chloroform extraction procedure and was precipitated with Na-acetate and 70% ethanol and resuspended in RNase – free water.
Details of DNase or RNase treatment	Genomic DNA was removed with the Turbo DNA free kit (Ambion® Thermo Fisher Scientific).
Nucleic acid quantification	Nucleotide concentrations were assessed on the Nanodrop ND-1000 spectrophotometer (NanoDrop® ND-1000, ISOGEN Life Science).
Purity	260/280 and 260/230 analysis
Reverse transcription	
Complete reaction conditions	cDNA was synthesized using Superscript TM III first-strand synthesis supermix (Thermo-fisher Scientific)
Amount of RNA and reaction	200ng of total RNA in a reaction volume of 20 µl
Storage condition of cDNA	cDNA was diluted to 1:9 in molecular water before storage at -20 °C
qPCR protocol	
Complete reaction conditions	SYBR Green Master Mix (Applied Biosystems)
Reaction volume and amount of cDNA	Reaction volume: 10 µl cDNA: 2.5 µl
Primer	0.3mM of forward and reverse primer
Polymerase, Mg2+. dNTP buffer	Included in SYBR Green Master Mix (Applied Biosystems, Thermo Fisher Scientific, US)
Complete thermocycling parameters	Universal cycling conditions: 10 minutes at 95^0 C 40 cycles: 15s at 95^0 C and 60s at 60^0 C
Manufacturer of qPCR instrument	ABI PRISM 7500 (Applied Biosystems)
qPCR validation	
Specificity (gel, sequence, melt or digest)	Samples with a melt temperature T_m deviating from the product specific T_m were excluded.
For SYBR Green I, Cq of the NTC	NTC's gave no amplification or at least 5 cycles higher than the highest sample Ct value
PCR efficiency	0.85 – 1.15
R^2 of calibration curve	≥ 97%
Data analysis	
qPCR analysis program (source)	qBase (Biogazelle)
Method of Cq determination	Hellemans et al., 2007
Results for NTCs	NTC's gave no amplification or at least 5 cycles higher than the highest sample Ct value
Justification of number and choice of reference genes	geNorm analysis
Statistical method for results	Non-parametric test (Kruskal-Wallis Test and post-hoc comparison test)
Software (source, version)	Prism GraphPad 8 (GraphPad, San Diego, US).

Table S2

Accumulation of Cu and Cd (µg gdw^{-1}) in regenerating head and tail fragments of *Schmidtea mediterranea* in different experimental groups after 7 days of exposure. Values are the average ± standard error of mean (SEM) of six independent biological replicates. A non – parametric test (Kruskal – Wallis and post-hoc comparison test), based on ranking was used to compare the treatment and control groups. *p<0.05.

dw = dry weight of the tissue

Head fragments | 7 days exposure				
Metals	Control	20 µM Cu	10 µM Cd	Cu+Cd
Cu	8.16 ± 0.63	26.62 ± 5.78*	8.58 ± 0.72	29.44 ± 4.14*
Cd	1.46 ± 0.05	1.5 ± 0.13	21.59 ± 0.95*	37.70 ± 1.03*
Tail fragments | 7 days exposure				
Metals	Control	20 µM Cu	10 µM Cd	Cu+Cd
Cu	23.48 ± 6.02	36.02 ± 7.66*	15.63 ± 1.16	33.72 ± 3.09 *
Cd	1.53 ± 0.13	1.38 ± 0.11	18.17 ± 1.33*	30.91 ± 2.43*

Table S3. Accumulation of Cu and Cd (µg gdw^{-1}) in adult *Schmidtea mediterranea* in different experimental groups after 7 and 14 days exposure. Values are the average ± standard error of mean (SEM) of six independent biological replicates. A non – parametric test (Kruskal – Wallis and post-hoc comparison test), based on ranking was used to compare the treatment and control groups. *p<0.05.

dw = dry weight of the tissue

Adult animals | 7 days exposure				
7D exposure	Control	20 µM Cu	10 µM Cd	Cu+Cd
Cu	23.35 ± 2.57	128.1 ± 12.59*	16.80 ± 0.54	55.22 ± 3.88
Cd	0.51 ± 0.05	0.90 ± 0.45	60.79 ± 2.97*	50.62 ± 9.87*
Adult animals | 14 days exposure				
14D exposure	Control	20 µM Cu	10 µM Cd	Cu+Cd
Cu	20.93 ± 3.01	85.06 ± 12.67*	13.22 ± 2.71	34.89 ± 8.17
Cd	0.41 ± 0.24	0.39 ± 0.05	62.96 ± 7.30*	42.15 ± 7.65*

Discussion

(Heavy) metals have been studied by many researchers due to their significant hazardous effects on human health and the environment (Hesse et al., 2018; Singh et al., 2022). Due to their toxic properties and ability to accumulate, they are considered a major source of environmental pollution. Industrialization, urbanization, and agricultural activities have led to an increase in metal concentrations in various habitats compared to their natural background levels (He et al., 2016). As a result, these do not occur singly but often as complex mixtures, resulting in concomitant exposure of organisms to multiple metals which influences the overall toxicity. The adverse effects of metals depend on the interplay between the chemical interactions with the other constituents in the exposure media and the interactions at the sites of uptake and toxic action (USEPA, 2007a, 2007b). As a result, the combined action of metal mixtures can have important consequences on metal risk assessment.

Already for a long time, the toxicity of metal (and other chemical) mixtures has been a subject of concern (Zhu et al., 2011; Ubani-Rex et al., 2017; Gao et al., 2018; Moyson, et al., 2018; 2019; Balali-Mood et al., 2021; Balistrieri et al., 2023; Wu et al., 2024), but there is still insufficient understanding concerning the general and metal-specific and/or metal mixture specific mechanisms of toxicity. In addition, knowledge of the developmental toxicity of metal mixtures in taxonomically distinct species is even less well documented and understood. Answering these questions can be useful for both risk assessment and prevention. In this context the present study was aimed to gain more insights into the molecular mechanisms underlying the effects induced by metals (Cu and Cd) singly and as a binary mixture, in taxonomically different species, representing distinct biological levels.

1. Metal accumulation does not always predict toxicity

Bioaccumulation is a process by which organisms accumulate substances, such as metals, or other chemicals from various environmental media, such as air, water, soil, sediment, and diet in their tissues over time (Alexander, 1999; SAB, 2006; Wang et al., 2016). Bioaccumulation occurs when an organism absorbs these substances at a rate that exceeds their ability to metabolize and eliminate them (Popek et al., 2018). This process involves several key steps, including, absorption (uptake), distribution, metabolism, and excretion

(ADME), and may occur passively or actively (Tsui & Wang et al., 2003; Luoma & Rainbow, 2005; Olguín & Sánchez-Galván, 2012). This phenomenon plays a crucial role in toxicological assessments of chemicals by determining the maximum concentration of chemicals in an organism's tissues relative to exposure levels, influencing toxicity levels (Daley et al., 2013; Abalaka et al., 2020). Studies have shown that the dynamics of bioaccumulation can vary depending on the species, metal type, and environmental conditions (Koelmans et al., 2013; Urien et al., 2015; Abalaka et al., 2020). The differences in the accumulation may be as a result of differences in assimilation, internal handling and elimination or most likely a combination of these (Rajeshkumar and Li., 2018). Therefore, Information on metal accumulation is important to understand the behaviour of metals in organisms and to assess the associated hazards (SAB, 2006; ECHA, 2017).

One of the important questions in the present work was to determine how accumulated metal levels influence the toxicity in different tissues and how this is affected when metals are combined. Metal accumulation was measured in all experiments, both after single and co-exposure of experimental animals to Cu and Cd. Overall, metal accumulation generally increased in the single exposure groups, but did not follow the same trend with mixture exposure when the results were compared for the different species. In zebrafish embryos (chapter 1), daphnia neonates (Chapter 2), and developing planarians (Chapter 3), aqueous exposure to Cu resulted in similar accumulation in both single and mixture exposed animals. In adult zebrafish and adult planarians (Chapters 1 and 3), Cu levels increased significantly during exposure to Cu singly, and showed a decrease in the presence of Cd, indicating inhibition by Cd. Adult daphnia (Chapter 2), showed consistent Cu accumulation when exposure occurred via the aqueous, dietary, and the combined pathway. The accumulation trends were also variable for Cd. In developing planarians (Chapter 3) and daphnia neonates (Chapter 2), higher Cd concentrations were observed in animals exposed to a Cu and Cd mixture than the animals exposed to one metal at a time via the direct exposure medium (aqueous). The opposite trend was observed in zebrafish embryos (chapter 1) and daphnia neonates (dietary and combined exposure) (Chapter 2). Here, Cd accumulation was higher compared to Cu when alone indicating inhibition by Cu. In adult zebrafish (Chapter 1), and adult planarians (Chapter 3), Cd accumulation was consistent between the animals exposed to a single metal and mixture, whereas in daphnia (Chapter 2), Cd levels increased only in single metal exposure groups. Overall, these results show the complexity of metal accumulation in three species, which needs to be further explored. For example, by

conducting longitudinal studies, to track accumulation over time in different species exposed to different concentrations, exposure durations, frequency and combinations of metals to reveal the kinetics of accumulation and potential interactions in different species.

Regardless of the accumulated metal levels in the tissues, toxicity was in general higher when animals were treated with the mixture of Cu and Cd. For example, a mixed exposure t to Cu and Cd resulted in distinctive lethal and non-lethal morphological changes, neuroregenerative impairments, altered behaviour, and a decline in survival in planarians exposed to the mixture, which were less severe in single metal exposure scenarios. Likewise, a simultaneous exposure to Cu and Cd resulted in increased stress levels in both age groups of daphnia compared to single exposure. In zebrafish, both embryos and adults that were simultaneously exposed to both metals showed increased sensitivity. These results show that metal accumulation is not always a predictor of toxicity, given the internal dynamics leading to toxicity. We show that the toxicity of metals, particularly in the mixture exposure scenarios, may be influenced by factors other than the total metal load, such as metal bioavailability. In aquatic toxicology, bioavailability serves as a key measure of the rate and extent to which a substance reaches its site of action (Adams et al., 2020). In the field of chemical risk assessment, bioavailability has been shown to be a key determinant influencing actual exposure and the potential for adverse effects (Naidu et al., 2008; Adams et al., 2020). As such, evaluation of both external (environmental) and internal (toxicological) bioavailability is crucial for the evaluation of metal toxicity. External bioavailability refers to metals that are available for uptake by organisms and are influenced by factors such as solubility limitations, sorption to solids or humic substances and distribution into inaccessible phases such as minerals (Adams et al., 2020). Other factors that influence the bioavailability of metals include the pH of the exposure medium, speciation, and the presence of chemicals that compete for binding sites, such as calcium, sodium or magnesium (Adams et al., 2020). The Biotic Ligand Model (BLM), a commonly used tool in aquatic toxicology, is particularly important for the assessment of the external bioavailability of individual metals (and for metal mixtures) in the aquatic environment (USEPA, 2007c; Smith et al., 2017; Crémazy et al., 2019). The BLM integrates the principles of metal speciation and competition for binding sites on biological ligands, such as dissolved organic matter and biological surfaces, to estimate the bioavailable fraction of metals in water. So far BLMs have been developed and validated for single metal exposure (e.g. Cu, Cd, Ni, Zn, Pb) but application to mixtures is more challenging and still under development.

Internal bioavailability refers to the proportion of metals that are absorbed by the body and interact with physiological receptors to produce toxic effects (Rainbow and Luoma, 2011; de Paiva Magalhães et al., 2015). It is organism-specific and is influenced by factors such as the number and type of binding sites at the site of action (Adams et al., 2020). We did not investigate bioavailability as it was not the subject of our study, making it challenging to determine whether the observed results were from total metal concentrations or the bioavailable fraction. However, studies have shown that the presence of one metal can alter the uptake of another metal, hence toxicity (Komjarova and Blust, 2008, 2009; Komjarova and Bury 2014; Delahaut et al., 2020). Furthermore, upon analyzing our results, we acknowledge the need for further investigation, particularly to understand the reasons for increased effects in co-exposures to Cu and Cd, despite similar or lower accumulations compared to single exposures. Therefore, further studies should be directed towards determining the extent to which metals in a mixture scenario become bioavailable, and how this affects their toxicity. Studies have indicated metal interactions to be species-specific and may not align with theoretical predictions based on current toxicological knowledge (Komjarova and Blust, 2009; Brix et al., 2017; Crémazy et al. 2019). Therefore, studies on metal mixtures need to focus on multiple species to compare metal bioavailability and determine if similar patterns or responses emerge.

2. Interactive toxicity of Cu and Cd is detrimental to early life stages

The potency of a toxic substance can be influenced by several factors related to the chemical, the environment, and the species. Differences in structural and functional organisation may alter the interaction between the organism and the toxicant by modifying the rates of absorption, distribution, excretion, and detoxification. Among the important factors related to the species is its life-stage. Most of the literature shows that early stages are more sensitive to the toxicants compared to adults (Mohammad, 2013; Holan et al., 2018; Majid et al., 2022). At these sensitive life stages, individual metals or their mixtures can cause permanent damage even at low levels of exposure that would have little or no effect on adult organisms. Metals are known to damage the organisms in their early life stages due to their higher sensitivity, that can probably be attributed to factors, such as surface area/volume ratio, under developed homeostatic mechanism to deal with the

toxicants, high skin permeability and presence of less fat than adults (metals may be sequestered in fat tissue, preventing them from reaching target organs) (Hoang et al., 2004; Hoang and Klaine, 2007; Mohammed, 2013; Attia et al., 2022). Developmental toxicity manifested in the form of altered survival, morphological abnormalities, altered growth, functional and behavioral deficits are often expressed as possible endpoints for evaluating the potential risks on development (USEPA, 1991; Morris et al., 2022; Xiong et al., 2022). In this study, we assessed the developmental alterations in zebrafish embryos (Chapter 1), neonates of daphnia (Chapter 2) and regenerating planarians (Chapter 3).

Planarians are known for their regenerative capacity and are recognized as a valuable *in vivo* animal model for studying the effects of chemical exposure. They provide a variety of quantifiable morphological, behavioral, and molecular endpoints that can be used to evaluate the effects of toxicants such as metals on various aspects of development (Hagstrom et al., 2016). They can synchronize their body size and modulate it according to environmental changes (Oviedo and Alvarado, 2003). One of the major questions of this thesis was to compare the effects of single and co-exposure to Cu and Cd on neuronal development in addition to other important aspects of developmental toxicity (such as impaired growth). Planarians are one of the simplest organisms to cephalize (the process in which sense organs, mouth, and nervous system concentrate towards the anterior side of the body, producing head) and have a unique ability to regenerate their entire central nervous system (CNS) following tissue loss, damage, or asexual reproduction (Cebrià 2007; Hagstrom et al., 2016). In asexual planarians, regeneration is the only mechanism of neurodevelopment. This complex process of neuroregeneration involves many of the same processes that occur during vertebrate neurodevelopment including stem cell migration, proliferation and differentiation and axonal guidance (Umesono et al., 2011; Hagstrom et al., 2016). Another important aspect of planarians is their place in the ecosystem. In contrast to other invertebrates (which are usually decomposers or primary consumers). Freshwater planarians are predators that assume the role of secondary consumers that mainly feed on other aquatic invertebrates (Wu & Li, 2018). Collecting toxicity data from organisms located in multiple nodes of the food web is of great value to ecotoxicologists and national environmental protection agencies for the comprehensive assessment of the impact and risk of environmental chemicals on the aquatic ecosystem.

Our results show reduced survival, reduced blastema and whole-body size and greater tissue malformations in the animals exposed to the mixture, which indicates the strong toxicity of a combined exposure to Cu and Cd. Nevertheless, some (less severe) effects were also seen in the individual metal exposure. Developmental impairment in form of growth reduction was also shown in daphnia. The developmental defects in zebrafish embryos were evident by the partial or complete trapping of embryos in chorion (egg envelope), because of which they either failed to hatch or delayed hatching. In chapters 1 and 3 of this thesis, we evaluated locomotor behavior as a functional endpoint in planarians and zebrafish embryos respectively. Alterations in functional competences such as locomotor behavior are usually reversible but are generally considered adverse. In this study, stronger behavioral alterations were demonstrated by planarians and zebrafish embryos in response to mixed treatment of Cu and Cd. In zebrafish behavioral changes were implicated by dysfunctional locomotor activity whereas in planarians impairment in body shapes was an additional response together with reduced locomotor behaviour. Taken together, metal mixtures were more potent in inducing developmental damages than the single metals. More importantly the combined toxicity was more than the sum of their individual effects (Chapter 2).

Some of the developmental impairments were common to all three species, such as decreased survival and growth in response to the mixtures, while others such as tissue malformations occurred only in planarians (lesions, lysis, blisters, bloating, contracted body, outgrowths, tissue regression) and zebrafish embryos (oedema; no eye development, head, and tail malformation). This difference can be attributed to the presence of an exoskeleton (cuticle) that acts as a barrier against diverse environmental threats including chemical damage (Moret and Moreau, 2012; Ritschar et al., 2020). While our study did not aim to determine which metal dominates the interaction in co-exposure scenarios, leading to cellular stress and resulting defense responses, the observed results, particularly related to morphological and physiological alterations in the developing animals suggest a significant influence of Cu. The effects were mostly common between Cu (alone) and mixture exposed animals, in the three tested species, indicating that the effects were primarily driven by Cu. This could possibly due to higher bioavailability of Cu, although a direct assessment of this parameter was not conducted. To address this question, further studies need to be carried out to determine bioavailability of Cu in a mixture scenario. Overall, our results show that exposure to Cu and Cd mixtures results in more severe developmental damage than exposure to individual metals, with effects ranging from decreased survival and growth

across all three species to more specific tissue malformations in planarians and zebrafish embryos.

3. Interactive toxicity of Cu and Cd elevates stress and defence responses

The cellular functioning of a cell depends on a coordinated action of several molecular processes involving important macromolecules such as DNA, RNA, proteins, and lipids. One of the major challenges of the cells is to deal with various internal and external alterations, some of which induce stress. Cellular stress is a result of potentially harmful extracellular and resulting intracellular changes. Cells respond to the stress by sensing the damage, transducing the signal, selecting the appropriate pathway, and executing the decision to repair or reprogram (Chen et al., 2010a) and adopt an altered state in the new environment. Severe stress can lead to cell death as cells are unable to cope up with the damage. Whether cells prompt a protective or destructive stress response largely depends on the nature and duration of the stress and on cell type (Fulda et al., 2010). Moreover, it is often the interplay between the cellular responses that ultimately determine the fate of the stressed cell. For example, protective responses such as the heat shock response or the response to unfolded proteins cause an increase in the activity of chaperone proteins that enhance the cell's ability to fold proteins, thus counteracting stress and promoting cell survival (Fulda et al., 2010; Bakhtisaran et al., 2015).

Studies have shown that metals can activate cellular signaling pathways that deregulate cellular pathways and subsequently induce toxicity (Fitsanakis and Aschne, 2005; Florea and Busselberg, 2006; Thévenod and Lee, 2013; Fatema et al., 2021). In this thesis, evaluation of metal – induced stress was performed by analysing the underlying cellular stress events via transcriptional profiling in all animal models. The mechanism of toxic action for both invertebrate and vertebrate models appeared largely similar and was mediated by the generation of reactive oxygen species, (Ercal, 2001) that led to the activation of antioxidant defense system (see Table 1). The upregulation of antioxidative genes (Chapter 1-3), the increase in H_2O_2 levels, alterations in the glutathione content (Chapter 2, & 3) and the expression of metallothionine indicated a strong reaction against oxidative damage in the mixture scenario. Toxicity was demonstrated by impaired growth, development and neuroregeneration in regenerating planarians. Similar interactive effects were observed in mixture-exposed zebrafish embryos (Chapter 1) and the disruption of antioxidant defenses was shown to be the underlying mechanism for morphological,

developmental, and behavioral endpoints. A similar conclusion was drawn from the experimental results in daphnia (Chapter 2) where disruption in cellular homeostasis led to the increase in toxicity that impaired the growth in neonates and reproduction in adults. Overall, the mode of action for both invertebrate and vertebrate models appeared largely similar, mediated by the generation of reactive oxygen species and subsequent activation of the antioxidant defense system. This suggests that the metals (Cu and Cd) are acting through similar pathways, supporting the idea of a common mechanism of action, which aligns with the CA model. But some of the effects on gene expression could not be explored because of the technical difficulties. We could not find a clear pattern to show which metal caused the observed antioxidant defense responses. Therefore, to address this issue, a deeper understanding of the contribution of each metal to toxicity in the scenarios involving metal mixtures is required, for example by comparative transcriptomic analysis between organisms exposed to different metal mixtures to identify mixture specific effects on antioxidant defense mechanisms.

Table 1. Response of antioxidant genes to Cu, Cd and their co-exposure in three tested species. Positive "+"sign indicates alterations in antioxidant gene activity and negative " – sign" indicates no activity.

Adult stages		Control	Copper	Cadmium	Mixture
D. rerio	Gills	-	-	-	+
	Liver	-	-	-	+
	Gut	-	-	+	+
D. magna	Aqueous exposure	-	-	-	-
	Dietary exposure	-	-	-	+
	Combined exposure	-	-	-	+
S. mediterranea		-	+	-	+
Developing stages		**Control**	**Copper**	**Cadmium**	**Mixture**
D. rerio		-	-	-	+
D. magna	Aqueous exposure	-	+	+	+
	Dietary exposure	-	-	-	+
	Combined exposure	-	+	+	+
S. mediterranea	Regenerating head	-	+	+	+
	Regenerating tail	-	-	+	+

4. Exposure pathways are crucial in toxicity assessment

Metal exposure is a fundamental issue for ecotoxicological assessment of aquatic organisms. When present in an aquatic environment, they disperse in the water column, remain in

solution or accumulate in sediments and/or biota (Frémion et al., 2016). Aquatic organisms can take up metals from both aqueous and dietary sources, yet it is commonly assumed that toxicity occurs primarily through aqueous exposures, on the assumption that metals are primarily taken up from the dissolved phase when exposure levels reach toxicity thresholds. Uptake from these sources depends on the concentrations and bioavailability of these metals. Once these metals enter the organism, they may follow different internal pathways leading to different levels of accumulation and toxicity (De Schamphelaere et a., 2004; Sofyan et al., 2006; Wen-Xiong, 2012). However, not much attention has been paid to the potential importance of combining both exposure pathways. Therefore, an important question in our study was whether aqueous and dietary exposures would have similar effects. In addition, it was also of particular interest to know the outcome of these exposures under their combined set-ups. In chapter 2 of this thesis, we tested adults of *D. magna* under different exposure (aqueous, dietary, and combined aqueous-dietary) settings to understand the relative contribution of each exposure pathway following exposure to Cu and Cd individually and as a mixture. The results showed higher potency of the combined exposure pathways in eliciting toxic effect, which were significantly stronger in Cu and Cd mixture treatments (more than additive) than either in aqueous or dietary exposures alone. For example, the Cu and Cd mixture treatment via the combined exposure pathway caused a stronger decrease in reproductive capacity of adult daphnids as well as induction of higher number of genes. The joint effects of combined aqueous and dietary exposures have previously been shown to induce stronger effects (Wilding and Maltby, 2006). We additionally tested daphnia neonates to distinguish between the effects of different exposure pathways during the developing stages. The results of these experiments also showed differential transcriptional responses under each setting compared to adults. For example, most of the genes that were activated upon aqueous and /or dietary exposure, were also observed upon combined exposure. Taken together, the results suggest that toxicity depends on exposure pathways and both aqueous and dietary exposure can contribute to the overall toxicity. Furthermore, for the development of water criteria, combined exposure to aqueous and dietary metal concentrations may be more appropriate, because it is considered more ecologically realistic.

5. Metallothionine a key player in mixture toxicity across three species

Studying metallothionein (MT) expression is important for understanding metal toxicity, for their known role in metal detoxification and homeostasis (Ruttkay-Nedecky, et al., 2013; Donald et al., 2015; Calvo et al., 2017). In our study, we investigated the expression of MT in response to exposure to Cu and Cd, both individually and in combination in the tested species. Our results showed a consistent alteration in the activity of MTs in the three species exposed to the Cu + Cd mixture. This increase in MT expression was also observed in some conditions with a single exposure to Cu or Cd (see Table 2). However, the most pronounced alterations occurred with co-exposure to Cu and Cd, indicating a stronger effect of the mixture on MT expression levels in different species and exposure routes. The upregulation specifically in response to Cu and Cd exposure suggests a cellular defence mechanism aimed at sequestering and detoxifying excess metal ions. The consistent upregulation of MTs across all tested species in response to mixture exposure implies its reliability as a sensitive biomarker for mixture-induced stress, regardless of species differences. Previous studies have also highlighted the potential of MT as a biomarker for assessing the toxic effects induced by metals such as mercury (Hg), Cd, Cu and zinc (Zn) (Sørmo et al. 2011; Saad et al., 2016; -Purać, et al., 2019).The comparative analysis of MT in our study provides further context to the utility of MT as a broad-spectrum biomarker for metal toxicity assessment and incorporating MT expression analysis into routine environmental monitoring protocols.

Table 2. Response of metallothionein to Cu, Cd and their co-exposure in three tested species. Positive "+"sign indicates alterations in gene activity and negative " – sign" indicates no activity.

Adult stages		Control	Copper	Cadmium	Mixture
D. rerio	Gills	-	+	+	+
	Liver	-	-	-	+
	Gut	-	+	+	+
D. magna	Aqueous exposure	-	-	+	+
	Dietary exposure	-	-	-	-
	Combined exposure	-	-	-	+
S. mediterranea		-	-	-	+
Developing stages		**Control**	**Copper**	**Cadmium**	**Mixture**
D. rerio		-	+	+	+
D. magna	Aqueous exposure	-	+	+	+
	Dietary exposure	-	-	-	-
	Combined exposure	-	+	+	+
S. mediterranea	Regenerating head	-	-	-	+
	Regenerating tail	-	-	-	+

6. Species-Specific Metal Sensitivity: Identifying ideal candidates for toxicity testing

The selection of appropriate species for toxicity testing is a critical aspect of both environmental or human health risk assessment. Species Sensitivity Distributions (SSDs) are often employed in such assessments to understand the range of sensitivities among different species. While there is no universally ideal species for toxicity testing, each species offers unique advantages and limitations that must be considered. Some species may be more suitable for certain types of toxicity assessments due to their physiological characteristics, while others may exhibit greater sensitivity to specific contaminants (Guilhermino et al., 2000; Boyd et al., 2003; Harwood et al., 2014; Wu et al., 2020; Kumar et al., 2020). When selecting a species for toxicity testing, it is essential to align the choice with the research question and ensure comprehensive coverage of endpoints. Moreover, cross-validation across species from different biological levels is necessary to gain a deeper understanding of the observed effects and underlying mechanisms. In this context, we investigated toxicity responses to single and co-exposure to Cu and Cd in three taxonomically different animal models. On comparing the three experimental animals, daphnia appeared to be more sensitive to metal stress, being affected at much lower concentrations (20% of 48h EC_{50} for Cu =0.25 µM and Cd = 0.25 µM) than zebrafish (25% of the 240-hour LC_{50} for Cu = 0.80 µM Cu and Cd = 0.25 µM Cd) and planarians (25% of the one week LC_{50} for Cu = 20 µM and Cd = 10 µM). Despite differential sensitivity, a similar molecular mechanism underlying toxicity, namely oxidative stress was activated. As these results are cross-validated among three species, despite distinct sensitivities, this increases chances of extrapolating the findings to a broader environmental and human health context, hence, the predictive power of assessment. However, it is important to acknowledge some limitations of our study. First, the selection of only three species may not fully represent the diversity of organisms in the environment. Furthermore, it was not possible to generate and/or position the sensitivity of the three species under investigation due to the significant time and resources required to gather data on other species. While it is feasible to compare these species based on acute and chronic toxicity results (position on an SSD), molecular-level comparisons are more challenging. This difficulty arises because gene expression and related data are much less documented for different species and comparable scenarios. Future research could expand the scope to include more species to better assess potential risks to human health. In addition, testing different chemical mixtures, such as a combination of metals with pesticides, pharmaceuticals,

industrial chemicals that are of wider occurrence would provide a more comprehensive understanding of the effects of metal exposure. This can further improve the reliability and applicability of toxicity testing approaches.

7. Implications in the European regulatory context and future directions

In the EU, assessment of chemical mixtures represents a major challenge to both scientific community and regulators, as there are still knowledge gaps in our understanding (Bopp et al.,2019. A key area where there is insufficient data is the extent and frequency of human and environmental exposure to specific chemical combinations and how this exposure may change over time. In addition, knowledge of the mode of action (MOA) of many chemicals remains inadequate. Currently, there is neither a generally accepted inventory of MOA nor a set of criteria for characterising or predicting modes of action, especially for data-poor chemicals. Furthermore, there is no consensus on how to categorize chemicals into assessment groups, which adds to the challenge (SCHER, 2012). In ecotoxicology, the complexity of the subject is even greater. Obtaining a comprehensive understanding of all potential MOA's in different organisms within a biological community is extremely difficult, if not impossible. In addition, ecologically relevant endpoints tend to be broader and less specific compared to human toxicology, making the assessment process even more complex (SCHER, 2012). Therefore, it is imperative to determine the MOA for all relevant taxonomic groups in aquatic and terrestrial ecosystems. However, the availability of such information is often limited. In addition, modes of action that appear different at the individual level may affect the same endpoint at the population level, necessitating the use of dose/concentration addition models to predict population-level effects. Furthermore, the choice of the independent effects approach may reduce some uncertainties, but may underestimate the combined effects of similarly acting chemicals. The current study cannot completely close the gap in our understanding, it adds valuable insight to the discussion on mixture effects. Furthermore, by identifying similarities in MOA in taxonomically distinct species exposed to metal mixtures, our study has shed light on a previously understudied aspect of mixture toxicity assessment. In addition, our research has emphasised the importance of exposure pathways in mediating the toxicity of metal mixtures. Although this aspect does not cover all complexities in the assessment of chemical mixtures, it provides a starting point for further research and refinement of assessment methods.

Conclusion

Metal pollution poses a significant health and environmental challenge, necessitating comprehensive investigation. The existing regulatory framework for toxicity testing primarily focus on the exposure to individual metal exposures, due to practical and technical limitations. Nevertheless, organisms in their natural habitats are usually exposed to a mixture of metal species. Recent shifts in focus on metal mixtures emphasise the importance of a more comprehensive evaluation of potential toxic effect of mixtures. While efforts have been made to investigate the toxicological effects of metal mixtures, studies targeting the molecular mechanisms and the modes of action in taxonomically distinct species with different sensitivities remain limited. This study aimed to address the existing gap in the litrature by studying the molecular mechanisms underlying the toxicity of Cu and Cd mixture in taxonomically different species, manifesting different sensitivities across different life-stages. Through comparative toxicity assessments in invertebrate *(Schmidtea mediterranea and Daphnia magna)* and vertebrate *(Danio rerio)* aquatic animal models at early and adult life-stages, our findings indicate that the Cu and Cd mixture exerted stronger effects (more than additive) across all tested species compared to the effects produced by either of these metals alone. This is evidenced by pronounced physiological, cellular, and molecular alterations in response to mixture stress in tested species. From a toxicodynamic standpoint, our findings reveal that the toxicity of metal mixtures can trigger similar mechanisms in different organisms, namely oxidative stress, irrespective of their taxonomic classification. Importantly, our results show that metal accumulation is not necessarily a predictor of toxicity, emphasizing the importance of studying internal dynamics leading to toxicity. Our results identify oxidative stress as a common underlying mechanism of toxicity, leading to morphological, physiological and /or behavioral perturbations, irrespective of accumulated metal levels. More specifically, exposure pathways were shown to significantly enhance toxicity, as evidenced by the more than additive effects of combined exposure pathway in daphnia adults and neonates. We also highlighted the higher vulnerability of early life-stages than adults. Taken together, our study provides useful insights into the dynamics of toxicity caused by metal mixtures.

Future perspectives

Current global chemical policies and risk management strategies primarily focus on the safety of individual chemicals ensuring that their levels remain below thresholds associated

with known adverse effects (Drakvik et al., 2020). However, there is a growing understanding that chemicals may produce joint toxicity even at low concentrations due to potential additivity or synergism of the individual components, leading to significant adverse effects on human health and the environment. Assessing the effects of chemical mixtures requires an integrated and systematic approach and close collaboration between different scientific fields, in particular toxicology, epidemiology, exposure science, risk assessment and statistics, to adequately integrate the data from all these disciplines. Currently, such integration is not comprehensively undertaken.

Our research into the combined toxicity of Cu and Cd in taxonomically different species has raised several new questions regarding the general, metal-specific and metal – mixture specific mechanisms of toxicity. While we compared the molecular and physiological responses to Cu and Cd single and mixture, a deeper understanding concerning mechanisms of toxicity need to be further explored. Particularly, developmental toxicity of metal mixtures in taxonomically distinct species remains poorly documented and understood, warranting further in-depth studies. To address these gaps, we propose three investigative categories;

1. exploration of cellular and molecular responses involved in different animal models in response to different metal combinations. Cellular and molecular responses could be elucidated utilizing techniques like transcriptomic (RNA-seq), proteomic, and metabolomic analysis
2. examination of tissue specific metal accumulation and toxicological bioavailability in a mixture context, and
3. investigation of subcellular partitioning of these metals in single and mixture treatments to reveal the mechanism behind the observed increased (additive and/or synergistic) effects.

While in our study experiments were conducted under controlled laboratory conditions, which potentially limits the environmental realism, further research should explore various toxicity endpoints (morphological impairment, neurotoxicity, developmental and reproductive toxicity, growth, and survival) under better developed environmental conditions, for example in a mesocosm setup. In addition, it is imperative to Identify and prioritise potentially relevant chemical mixtures for toxicity testing. However, given the almost infinite number of possible combinations of chemicals to which humans and

environmental species are exposed, it is important to prioritise potentially harmful mixtures. The following criteria proposed by EU Commission for identifying potentially harmful chemical mixtures offer a structured approach to prioritize substances of concern (SCHER, 2012): The focus should be placed on the chemicals:

- presenting significant exposure levels to humans and/or the environment, particularly those close to established health-based guideline values (HBGVs) or derived no effect levels (DNELs) or predicted no-effect concentration (PNEC).
- that are manufactured or marketed as multi-constituent substances or commercial mixtures containing several components or active substances. This encompasses substances governed by EU legislation such as REACH, CLP, pesticide and biocidal products legislation, and food legislation, reflecting regulatory recognition of their potential risk.
- with the likelihood of frequent or large-scale exposure within human populations or the environment.
- persistence of chemicals in the body and/or in the environment (persistent chemicals should be prioritised)
- known information of potential interaction at levels of human and environmental exposure.
- predictive information that chemicals act similarly such as (quantitative) structure activity relationships and structural alerts (QSAR).

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