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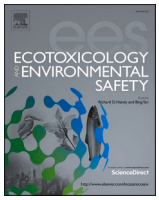

Insights into the combined toxicity of copper and cadmium in zebrafish (*Danio rerio*) embryos and adults

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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 in co-exposure scenarios and across different life stages. We investigated the toxic effects of copper (0.80 μM Cu) and cadmium (0.25 μM Cd), both individually and in combination, on zebrafish (*Danio rerio*) embryos and adults. Key morphological and functional endpoints were assessed in embryos at 96 hours post-fertilisation (hpf) and in adults after seven days of exposure. Metal accumulation and molecular responses related to oxidative stress, apoptosis, metal transport, and DNA damage were studied at both life stages to gain insights into general and specific stress responses. The results show increased sensitivity of both embryos and adult animals to the co-exposure compared to single metal exposures. Embryos displayed higher vulnerability compared to adults. Oxidative stress emerged as a common mechanism of toxicity across both life stages, albeit with distinct defensive responses. Our results challenge the simplified assumption that metal accumulation alone can predict toxicity, highlighting the necessity of considering internal metal dynamics and physiological resilience. Investigations into other metal combinations and their effects on diverse species are warranted to fully elucidate the complexities of mixture toxicity in organisms.

1. Introduction

Metal pollution is one of the major environmental concerns, largely driven by the widespread use of metals and their release from industrial activities, agriculture, and urbanisation, which leads to contamination of soil and water. Studies have shown that metal mixtures, such as cadmium (Cd), copper (Cu), zinc (Zn), nickel (Ni), and lead (Pb), can interact in ways that produce additive, synergistic, or antagonistic effects (Zeb et al., 2017; Moyson et al., 2018; Castaldo et al., 2020; Arreguin-Rebolledo et al., 2024). Despite advances in understanding of metal mixture effects, significant gaps remain in deciphering the molecular mechanisms underlying mixture toxicity. Furthermore, age-specific responses to metal mixtures are less explored, even though they are crucial for accurate ecological and human health risk assessments. Addressing these gaps is crucial for better understanding the developmental and physiological factors that drive toxicological responses in organisms. In the current study, we investigated the impact of Cu and Cd on adults and embryos of zebrafish (*Danio rerio*) under single and binary exposure setups. These metals were selected for their frequent occurrence in the environment, persistence, distinct biological roles, their well-documented adverse effects (e.g., essential versus non-essential) and their strong interactive effects (ATSDR, 2004; ATSDR, 2012; Kamunde and MacPhail, 2011; Sadeq and Beckerman, 2020). Their co-existence in polluted environments, particularly in areas affected by mining and industrial discharge, necessitates evaluating their combined toxicity, as their interactions can significantly influence bioavailability, uptake, and overall toxicity.

Copper is an essential element involved in various physiological processes (de Romaña et al., 2011; Taylor et al., 2020). It serves as a cofactor for key enzyme systems, including superoxide dismutase (SOD),

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ceruloplasmin, mitochondrial cytochrome-c oxidase, and tyrosinase, which are crucial for antioxidant defence, iron metabolism, and cellular respiration (Hefnawy and Khaiat, 2015). However, disruptions in Cu homeostasis, whether deficiency or excess, can lead to adverse health effects (Stern et al., 2007; Taylor et al., 2020). Elevated Cu levels are particularly associated with liver toxicity and neurological disorders (Taylor et al., 2020). Copper-induced toxicity is primarily linked to its ability to undergo redox cycling between oxidised (Cu^{2+}) and reduced (Cu^{+}) states, which facilitates the production of reactive oxygen species (ROS) through the Haber-Weiss reaction (Letelier et al., 2005; Stern et al., 2007). Excessive ROS generation can trigger oxidative stress, resulting in protein oxidation, DNA damage, enzyme inhibition, lipid peroxidation, apoptotic pathway activation, and ultimately, cell death (Gaetke et al., 2014; Lesiów et al., 2019; Villalpando-Rodriguez and Gibson, 2021).

Cadmium is a non-essential element that poses significant health risks (Genchi et al., 2020). It is classified as a human carcinogen (Group 1) by the International Agency for Research on Cancer (IARC, 2012). Cadmium exposure has been associated with nephrotoxic, genotoxic, and immunotoxic effects (Satarug, 2018; Nagaraju et al., 2022; Ebokaiwe et al., 2023). Additionally, it has been reported to exert strong teratogenic effects and negatively impact both male and female reproductive health (Kumar and Sharma, 2019; Massányi et al., 2020; Zhu et al., 2020; Liu et al., 2021). Cadmium-induced toxicity is linked to oxidative stress, disruption of essential metal homeostasis (such as Zn, & Ca), and interference with cellular functions (mitochondrial dysfunction) (Mao et al., 2011; Đukić-ćosić et al., 2020; Qu and Zheng, 2024). Furthermore, Cd exposure has been shown to interfere with DNA repair mechanisms, promote abnormal cellular proliferation, and inhibit apoptosis, contributing to its carcinogenic and toxic effects (Zarros et al., 2008; Rani et al., 2014; Genchi et al., 2020).

Zebrafish serve as an ideal model organism for toxicological and developmental biology research due to their genetic similarity to higher vertebrates, rapid development, and well-characterised stress response mechanisms (McCollum et al., 2011; Roper and Tanguay, 2018; Zhao et al., 2024). We hypothesise that developmental-stage differences influence metal toxicity, with zebrafish embryos and adults exhibiting distinct molecular signatures reflective of their physiological maturity and adaptive responses. This hypothesis is based on prior research demonstrating the synergistic toxicity of Cu and Cd in zebrafish and other aquatic 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 the well-established greater sensitivity of early life stages to environmental stressors (Mohammed, 2013; Majid et al., 2022). To determine the toxicity mechanisms, we examined key molecular events triggered by metal exposure in both embryos and adults. Our results reveal stressor-specific responses to single and binary metal exposures in both early and adult life stages, as well as distinct organ-specific responses in adults.

2. Materials and methods

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 therefore, did not require approval by the ECD.

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). Water conditions were maintained as per OECD test guidelines (OECD TG 236) OECD, 2013), with the following parameters: temperature (28 ± 1^0 C), conductivity (500 ± 25 µS/cm), hardness (30 mg/L $CaCO_3$), pH (7.5 ± 0.2), dissolved oxygen ($O_2 \geq 80$ % 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 < 0.25 mg/L, nitrite < 0.3 mg/L and nitrate < 12.5 mg/L) and were monitored twice weekly using Tetratest kits (Tera Werke, Melle, Germany). An automated 14 h light /10 h dark was maintained. The breeding stock was fed four times daily; 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 paired in a 2:1 ratio (OECD TG 236) and separated overnight in breeding tanks with a perforated bottom and dividers. Spawning and fertilisation occurred 45 minutes after lights were turned on. Eggs were then collected via disposable plastic tubing and transferred into clean reconstituted freshwater.

Exposures began at 0 – 2 hpf, as illustrated in Fig. 1. Molecular and physiological responses in zebrafish were evaluated using 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 ($CdCl_2{\cdot}H_2O$) were prepared in reconstituted freshwater and diluted to working solutions of 0.80 µM Cu and 0.25 µM Cd. These concentrations (selected for potential sublethal effects in both adults and embryos), were based on the established concentration range from Pilehvar et al. (2020) (see Section 2.3). To maintain 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 randomly divided into 5 groups: a negative control ($n = 40$), a positive control ($n = 20$), Cu exposure (0.80 µM Cu) ($n = 20$), Cd exposure (0.25 µM Cd) ($n = 20$) and a combined exposure (0.80 µM Cu + 0.25 µM Cd) ($n = 20$). Unfertilised, damaged, or irregular eggs were discarded. The exposure lasted 96 hpf, with fresh solutions renewed at 48 hpf. The entire experiment was repeated twice following OECD Test Guideline 236 (OECD TG 236). At 96 hpf, embryos from all treatment groups were sampled, washed with medium, and placed in 1.5 mL polypropylene centrifuge tubes. Excess water was removed, and samples were snap-frozen in liquid nitrogen. Metal accumulation samples were weighed and stored at −70°C. Samples for gene expression analysis were stored in RNAlater® solution (Ambion, Lithuania) overnight at 4°C and then transferred to −70°C until further analysis.

2.2.2. Zebrafish adults

Adult zebrafish (average fresh weight: 0.45 ± 0.10 g, average length: 2.0 ± 0.04 cm) were obtained from the University of Antwerp zebrafish facility and acclimated for 14 days in a well-aerated 100 L glass aquarium containing 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 CaCO3). After acclimation, fish were weighed and randomly transferred to polypropylene aquaria containing 7.5 L of exposure medium. Each aquarium housed 5 fish, with each exposure run in duplicate to obtain 10 individuals per treatment while minimising animal use. After 7 days of exposure, fish from both control and treatment groups were sampled, weighed, and euthanised by rapid cooling in melting ice (≤ 4°C). Organs were then dissected on ice, with gills, liver,

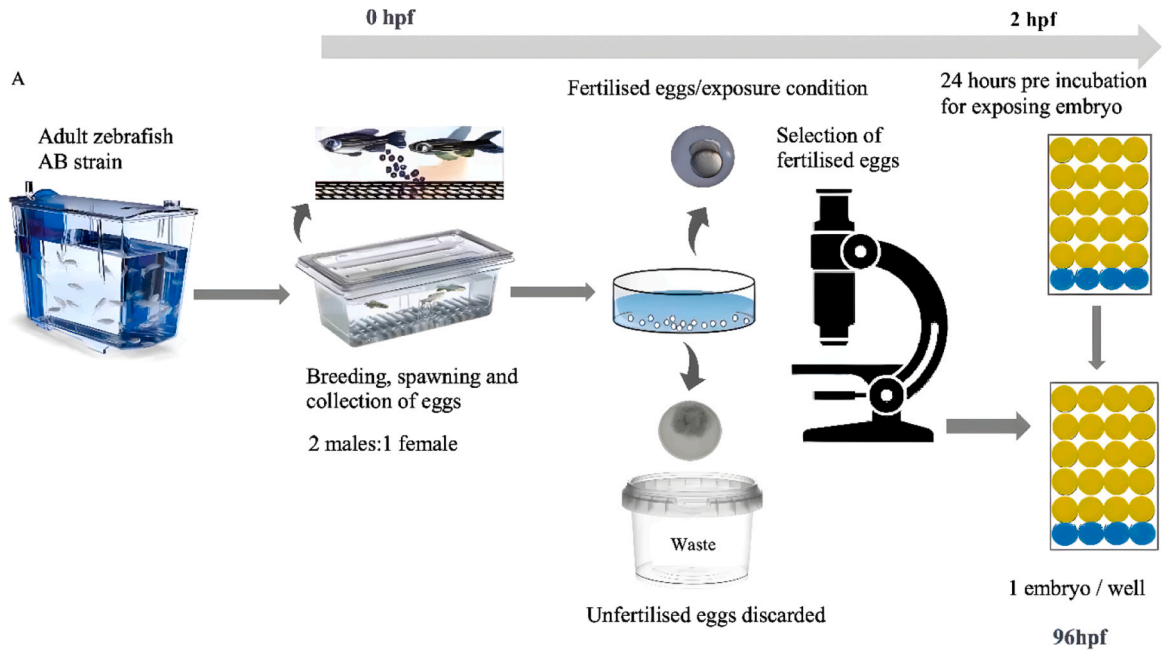

Fig. 1. Illustration of early life stage zebrafish acute toxicity test (from left to right): breeding, spawning, collection of eggs, selection of eggs using an inverted microscope and distribution of embryos over pre incubated 24 well plates prepared with respective exposure concentrations/control. n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

gut, and carcasses collected and divided into subsamples for metal and molecular analysis. Samples for metal analysis were weighed and stored at −70°C until use. Samples for gene expression were preserved in RNAlater® solution (Ambion, Lithuania) overnight at 4°C, then transferred to −70°C for further analysis. To minimise contamination, all surfaces and instruments used in the RNA extraction protocol were treated with RNase Away® (MBP Inc., San Diego, CA).

Throughout the experiments, zebrafish were maintained at a controlled temperature of 28°C under a 14 h light / 10 h dark photoperiod. Water was continuously aerated using an aeration line, and exposures were conducted under a semi-static regime, with 80 % water renewal every 48 hours using freshly prepared solutions. To assess Cu and Cd concentrations, water samples were collected from each tank before and after renewal, then filtered using an Acrodisc® 0.20 μm Supor Membrane syringe filter (Pall Life Sciences, Ville St. Laurent, QC). Samples were acidified with 200 μL of ultrapure nitric acid and analysed for dissolved Cu and Cd using inductively coupled plasma-mass spectrometry (ICP-MS 7700x, Agilent Technologies). Measured Cu and Cd concentrations remained within 94–100 % of nominal values, while metal concentrations in the control medium were below the method quantification limit (< 0.1 μg/L). Ammonia, nitrite, and nitrate levels were monitored every 24 hours using Tetratest kits (Tetra®, Melle, Germany) and maintained within optimal ranges for zebrafish (ammonia < 0.02 mg/L; nitrite < 0.1 mg/L; nitrate < 50 mg/L) (Pilehvar et al., 2020). During acclimation, fish were fed once daily with Sera Vipan® (Germany) at 1 % of fresh body weight. To prevent interference with metal uptake pathways and avoid increased ammonia production, no food was provided during the exposure phase.

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 obtaining an estimate of the EC10 and EC50 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 0.1–8 μM, and Cd concentrations in the range ca. 0.2–22 μM. The acute toxicity of Cu in adult zebrafish was much higher compared to Cd with respective LC50/LC10 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 was conducted according to OECD guidelines (OECD, TG 236) to evaluate the effects of metal exposure on embryonic development. Various morphological and physiological changes, as well as the survival rates, were assessed in developing embryos. Embryos were randomly distributed over 24 well plates (pre-saturated overnight with the test solutions) with one embryo and 2 mL of solution per well. A total of 20 treatment groups and 4 internal negative controls were used, with two replicate plates prepared for each treatment, including negative controls. Positive control plates containing 4 mg/L of 3,4-dichloroaniline (CAS 95–76–1, 98 % purity, Sigma-Aldrich) were included to compare sensitivity across groups (OECD TG 236). All plates were covered with Parafilm® (Parafilm® M, Bemis Europe, Soignies, Belgium) and maintained in an incubator (MIR-254-PE, Panasonic, TCPS, Rotselaar, Belgium) at a constant temperature of 28.5 ± 0.2°C with a 14/10 h light/dark cycle. Test solutions were renewed every 48 hours. Mortality and hatching were monitored daily, and a full morphological analysis was performed at 96 hpf using a Leica S8APO stereomicroscope (Leica Microsystems GmbH, Germany), as per OECD TG 236. Dead embryos and remaining chorions after hatching were removed every 24 hours. Lethal endpoints (e.g., non-detachment of tail, absence of somites, absence of heartbeat, lack of hatching), sublethal effects (e.g., impaired eye development, abnormal pigmentation, oedema, blood accumulation), and morphological malformations (e.g., head and tail deformities) were observed and recorded. Each abnormality was scored as either 0 (normal) or 1 (abnormal). For mortality assessment, four key endpoints were checked every 24 hours: lack of somites, failure of tail detachment, coagulation, and absence of heartbeat.

2.5. Morphological, physiological and survival analysis in adults

Morphological changes, including abnormal skin pigmentation and physiological abnormalities such as loss of equilibrium, abnormal swimming behaviour, ventilatory function and other visible abnormalities (as clinical signs) were visually monitored to detect deviations from normal body form and overall health, following OECD test guidelines (OECD TG: 203) (OECD, 2019). Observations were recorded at 24 hours interval throughout 7 days 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).

2.6. Behaviour analysis in embryos

The embryos used for teratogenicity analysis were also examined for behavioural 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 groups were used for analysis.

2.7. Behaviour analysis in adults

Behavioural abnormalities were assessed to detect deviations from normal behaviour, following 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, and over/under reactivity to stimulus was monitored.

2.8. Metal accumulation in embryos and adults

The accumulation of Cu and Cd was determined 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) was measured in all tissue samples to assess any alterations in their levels, considering their physiological significance and their interactions with Cu and Cd (Mebane, 2023; Liu et al., 2023). Tissue samples (10 samples per condition) were thawed, weighted, and dried at 60 °C for 24 hrs. The dried tissues were then digested with 0.5 mL ultra-pure nitric acid (Merck, Darmstadt, Germany) in a microwave using the following cycle: 100 watts (3-minutes x 3 times), 180 watts (3-minutes x 3 times), 300 watts (1-minute x 1 time). The 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 analyse 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). The 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 analyse the accumulation of metals. To validate the method, triplicate samples of reference standard mussel tissue (NIST-2976) and reagent blank solutions were prepared. Water samples (10 mL) were collected from each aquarium after every 24 h period, filtered through a 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 for all selected zebrafish genes were designed using the nucleotide sequences given on NCBI GenBank® (https://www.ncbi.nlm.nih.gov/nuccore). To optimise primer design across exon boundaries, primers were initially designed manually and then analysed using the primer analysis software LightCycler® probe design software (version 3.5, Roche Molecular Biochemicals, Germany). All the primer sequences are listed in Tables 1 and 2.

2.9.2. RNA extraction and cDNA synthesis

RNA was extracted using the phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). Snap frozen embryos and adult zebrafish samples were dissolved in 200 µL lysis buffer (Qiagen, catalogue number 79216) containing 1 % ß-mercaptoethanol and precipitated with Na-acetate and 70 % ethanol. RNA concentration and purity was determined spectrophotometrically using a Nanodrop ND-1000 spectrophotometer (NanoDrop® ND-1000, ISOGEN Life Science). Genomic DNA contamination was removed with the Turbo DNA free kit (Ambion® Thermo Fisher Scientific). Complementary DNA (cDNA) was synthesised using Superscript TM III first-strand synthesis supermix (Thermo-Fisher Scientific) following the manufacturer's instructions. The cDNA samples were stored at −20 °C until their use in real-time quantitative PCR analysis.

2.9.3. Real – time quantitative PCR analysis

Quantitative real-time qPCR (qPCR) was performed using optical 96-well plates (Applied Biosystems, Thermo Fisher Scientific) with Fast SYBR Green master mix (Applied Biosystems, Thermo Fisher Scientific) on a 7500 Fast Real-time PCR System (Applied Biosystems, Life Technologies). Primer efficiencies were evaluated by a four-point standard curve, prepared by a 1:3 serial dilution of cDNA. Selection of reference genes for the qPCR normalisation followed the method given by Vandesompele et al. (2002) and Tang et al. (2007). From tested reference genes (see Table 1), the three or four most stable genes, depending on tissue type in adults or embryos, were selected using GeNorm analysis. Gene expression analyses were conducted following the Minimum Information for Publication of Quantitative Real-Time Experiments (MIQE) guidelines (Bustin et al., 2009) and the methodology described by Rocha et al., (2016). Transcriptional profiles were examined for the genes involved in cellular redox state, the cell cycle and DNA repair mechanisms, structural development and metal transport were studied (see Table 2). Further procedural details are provided in Table S1 (see supplementary data).

2.10. Statistical analysis

Statistical analysis were conducted using GraphPad Prism 8.1.2 statistical software (GraphPad software, CA, USA). Each parameter was analysed separately in comparison with the appropriate controls. Data normality was tested using the D'Agostino-Pearson test. Based on normality distribution, statistical comparisons were performed using: parametric test (one-way ANOVA), when normality assumptions were met followed by Holm-Sidak's multiple comparison test; and Non-parametric tests (Kruskal–Wallis one-way ANOVA), when normality assumptions were not met, followed by Dunn's method for multiple comparisons. A p-value < 0.05 was 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
β2 m	Beta chain of major histocompatibility complex I molecule	NM131163.2	F: CCAAAGTAGCTGCTACAGG R: GCCAACAAGTGCAGAGT
ef1α	Protein translation	ENSDART00000023156	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 target genes 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) developed normally, whereas all the metal-exposed embryos exhibited different signs of toxicity. Some effects were lethal, such as lack of hatching and survival, while others were non-lethal and/or teratogenic, including morphological and physiological abnormalities such as oedema, blood accumulation (blood clogging), impaired eye

development, and head and tail malformations (see Fig. 2 and Table 3).

Zebrafish embryos hatch around 48 hpf and this process is completed by 72 hpf (Westerfield, 2000). Hatching was assessed at 48, 72 and 96 hpf. Compared to controls, hatching was significantly impaired in metal-exposed embryos. At 96 hpf 80 % of Cu-exposed embryos and 95 % Cu+Cd-exposed embryos failed to hatch. Cd exposure alone delayed hatching in 30 % of embryos at 72 hpf, whereas unexposed embryos exhibited 100 % hatching success. Mortality rates at 96 hpf were 10 % (Cu alone), 40 % (Cu+Cd), and 5 % (Cd alone), as embryos remained within the chorionic membrane without a detectable heartbeat or exhibited coagulation. The positive control 3,4-dichloroaniline (DCA) caused 70 % mortality at 72 hpf, increasing to 100 % at 96 hpf.

Metal-exposed embryos exhibited several teratogenic effects. Oedema was observed in 80 % of Cu+Cd-exposed, 60 % of Cu-exposed, and 5 % of Cd-exposed embryos, affecting both hatched and unhatched individuals. Among hatched embryos, spinal curvature was observed in 60 % (Cu+Cd), 20 % (Cu), and 5 % (Cd) of exposed groups. Blood accumulation in the tail region was found in 20 % of Cu-exposed, 5 % of Cd-exposed, and 30 % of Cu+Cd-exposed embryos. The effects on eye development and head malformation were specific to 5 % of Cu+Cd-exposed embryos and were not observed in other groups (see Table 3).

Embryo behaviour was assessed by examining the spontaneous movements inside the chorion and the swimming ability at 96 hpf days. Spontaneous movements were absent in 60 % of the Cu-exposed and 70 % of Cu+Cd-exposed embryos, whereas Cd-exposed embryos and the control group showed normal movements. Swimming ability was completely absent in Cu+Cd-exposed larvae, while Cu- and Cd-exposed larvae were minimally affected (see Fig. 3).

3.2. Assessment of metal effects in adults

Adults exposed to Cu and Cd alone showed no morphological, physiological or behavioural abnormalities. However, 20 % of Cu+Cd-exposed adults exhibited hypoactivity and mild abdominal swelling (oedema), with both symptoms occurring in the same individuals. Survival rates remained unaffected across all exposure groups (see Table 4).

3.3. Metal accumulation in embryos and adults

The accumulation of Cu, Cd, Fe, Zn, Na, K, Ca, and Mg was assessed in embryos and adults following exposure to Cu and Cd alone and in combination. In embryos, Cu levels remained unchanged following both Cu exposures and Cu+Cd exposure. Cadmium accumulation was higher in Cd-exposed embryos, followed by those exposed to the Cu+Cd mixture (see Fig. 4A). Analysis of essential metals revealed a significant decrease in Na and Ca levels in embryos exposed to Cd+Cu compared to control (see Table S2 in supplementary data).

In adults, metal concentrations were measured in the gills, liver, gut, and carcass (muscle and bones) (see Fig. 4B for Cu and Cd; Table S3 for other metals). Cu concentrations increased significantly in all tissues when the animals were exposed to Cu alone. In the animals exposed to Cd+Cu, the Cu levels increased as compared to the unexposed group but were lower compared to Cu alone. Cd accumulation was comparable across the Cd-only and Cu+Cd exposure groups in all three organs. Increased Ca and Mg levels were observed in the gills of Cu-exposed adults and the liver of Cu+Cd-exposed adults. Zn concentrations were significantly elevated in the liver of Cu+Cd exposed adults and remained unchanged in single-metal exposures.

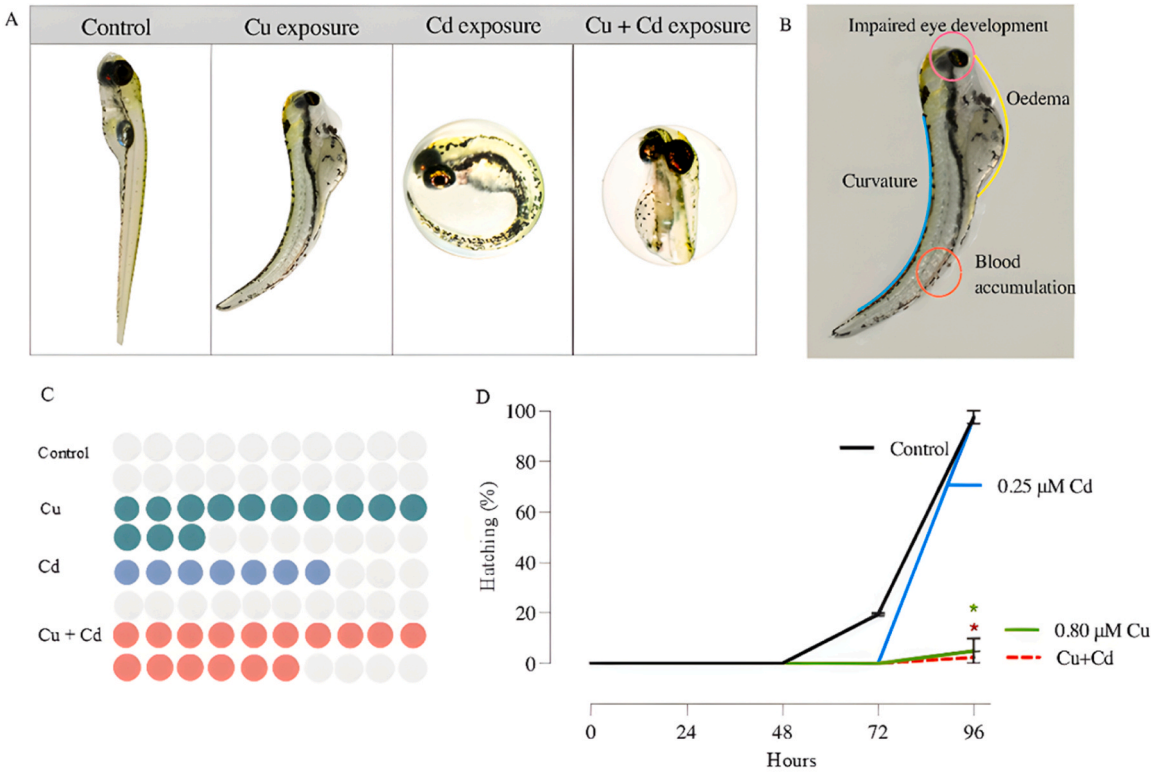

Fig. 2. Morphology and Development of zebrafish embryos. (A) Microscopic images of teratogenic effects in zebrafish embryos exposed to 0 µM (control), 0.80 µM Cu, 0.25 µM Cd and the Cu+Cd co-exposure at 96 hpf. (B) Illustration of malformations in hatched zebrafish embryos (impaired eye development, tail curvature, oedema, blood accumulation). (C) Visualization of zebrafish embryo hatching at 96 hpf. The colours represent hatching failure in Cu (green), Cd (blue) and Cu+Cd (red). Grey colour represents hatching success. (D) Graphical representation of hatching rate of zebrafish embryos in exposed and control groups at various 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. *$p < 0.05$.

Table 3
Overview of lethal and sublethal 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 (hours)																
	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 detachment		0	0	0		0	0	0		0	0	0		0	0	0
Lack of somite formation		0	0	0		0	0	7		0	0	3		0	10	30
Lack of heartbeat																
Sublethal																
Lack of hatching		0	0	0		100	80	80		30	30	0		100	95	95
Impaired eye development		0	0	0		0	0	0		0	0	0		0	0	5
Deviating pigmentation		0	0	0		0	0	0		0	0	0		0	0	0
Oedema		0	0	0		0	0	60		0	0	5		0	0	80
Blood accumulation		0	0	0		0	0	20		0	0	5		0	0	30
Head malformation		0	0	0		0	0	0		0	0	0		0	0	5
Tail malformation		0	0	0		0	20	20		0	5	5		0	60	60

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.

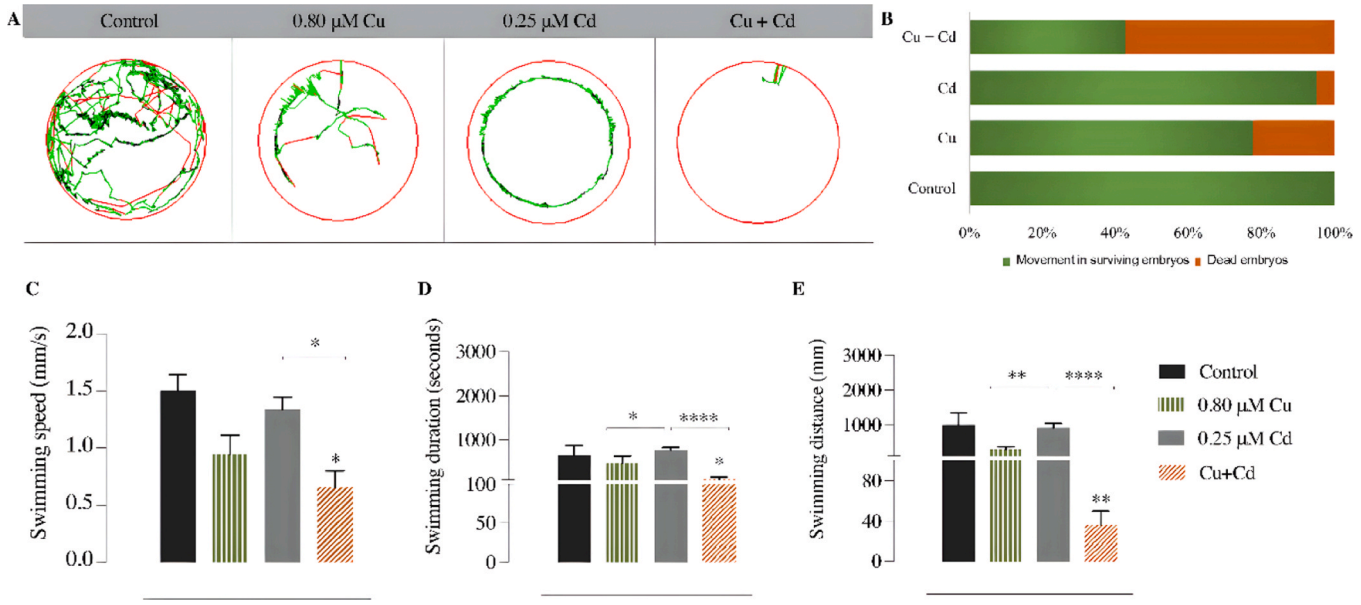

Fig. 3. Behavioral analysis of zebrafish larvae: (A) Representative video tracks of zebrafish larvae exposed to 0 μM (control), 0.80 μM Cu, 0.25 μM Cd and Cu+Cd at 96hpf. (B-E) effects of single and combined exposure of Cu and Cd at 96hpf on: (B) movement in surviving embryos (C) swimming speed (mm/s), (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. *$p < 0.05$, * *$p < 0.01$, * ** *$p < 0.0001$.

3.4. Gene expression analysis in embryos and adults

In embryos, exposure to the Cu+Cd mixture led to a significant decrease in the expression of antioxidant genes, including glutathione reductase (gsr), glutathione transferase (gstm), and superoxide dismutase (sod1), while no significant changes were observed under single exposure conditions (see Fig. 5). Cu exposure alone and Cu+Cd exposure both resulted in upregulation of the DNA repair gene (gadd45ba) and the calcium transport gene (ecac), with no changes in the Cd-only group. Copper exposure also led to an upregulation of the apoptosis-related gene (bcl2). The heat shock genes (hsp70, hsp90), the neuronal gene (neuroD), the epithelial calcium channel (ecac) gene and metallothionein (mt) exhibited altered expression under all exposure conditions. Specifically, mt and ecac were upregulated, while neuroD was downregulated.

In adults, exposure to Cu and Cd alone resulted in upregulation of ecac in gills, which remained unchanged in the Cu+Cd exposure group (see Fig. 6). The Cu+Cd exposure also led to changes in a broader range of genes. Specifically, the antioxidant genes gsr and sod1 were upregulated, while sod2 and sod3 were downregulated. The pro-apoptotic gene (bax) was upregulated, and the anti-apoptotic gene (bcl2) was downregulated under the combined exposure. DNA repair genes exhibited varying expression: rad51 was upregulated, while gadd45 was downregulated. Additionally, the heat shock gene hsp70 and the copper transporter gene (ctr1) were both upregulated. Metallothionein was upregulated across all three exposure conditions.

In the liver, changes in gene expression were observed only in response to the Cu+Cd exposure. Specifically, antioxidant genes such as catalase (cat), sod1, and sod2 were upregulated. The Cu+Cd exposure also induced upregulation of hsp70, mt, ctr1, and the zinc transport protein (zip1). In contrast, a downregulation of ecac was noted in the Cu+Cd-exposed group. Notably, the expression patterns of DNA repair

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 combined 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.

genes in the liver mirrored those in the gills, with upregulation of rad51 and downregulation of gadd45.

In the gut, the expression of three genes, cat, hsp70, and mt, was upregulated under both single and mixture exposure conditions. Cd exposure alone also led to upregulation of the antioxidant gene gsr, while Cu+Cd exposure resulted in the upregulation of sod3 and the apoptotic genes bcl2, alongside a downregulation of bax. The activity of DNA repair genes in the gut was consistent with those in the gills and liver.

4. Discussion

The present study aimed to investigate the mixture toxicity effects of Cu and Cd in zebrafish at both developmental and adult life stages. Our findings show the toxicity mechanisms triggered by Cu and Cd alone, as well as their combined exposure, with distinct responses observed in embryos and adults.

The exposure of zebrafish embryos to Cu and Cd resulted in a variety of toxic effects, including developmental delays, morphological deformities, and changes in behaviour. The most striking observation was the significant impairment of hatching, a critical event during embryogenesis in which the embryo emerges from the chorion and begins free movement. Despite the protective function of the chorion, metals can cross it and disrupt developmental processes (Wang et al., 2020). Notably, the combined exposure to Cu+Cd resulted in more severe effects compared to single metal exposures, which suggests a potential synergistic interaction between these metals. The failure of hatching and increased mortality rates in the Cu+Cd group may be attributed to the exacerbation of oxidative stress, a well-established mechanism of metal-induced toxicity (Gaetke et al., 2014; Zarros et al., 2008). Similar Cu-induced hatching impairments have been reported in various fish species, including zebrafish (Jezierska et al., 2009; Johnson et al., 2007; Zhang et al., 2018), while Cd exposure has been associated with delayed hatching at higher concentrations (Aldavood et al., 2020; Mitovic et al., 2021) (see Table 3). Hatching impairment is often associated with biochemical disruptions, such as reduced synthesis of hatching enzymes by the hatching gland, which are essential for chorion digestion (Fraysse et al., 2006). Other factors contributing to hatching inhibition include

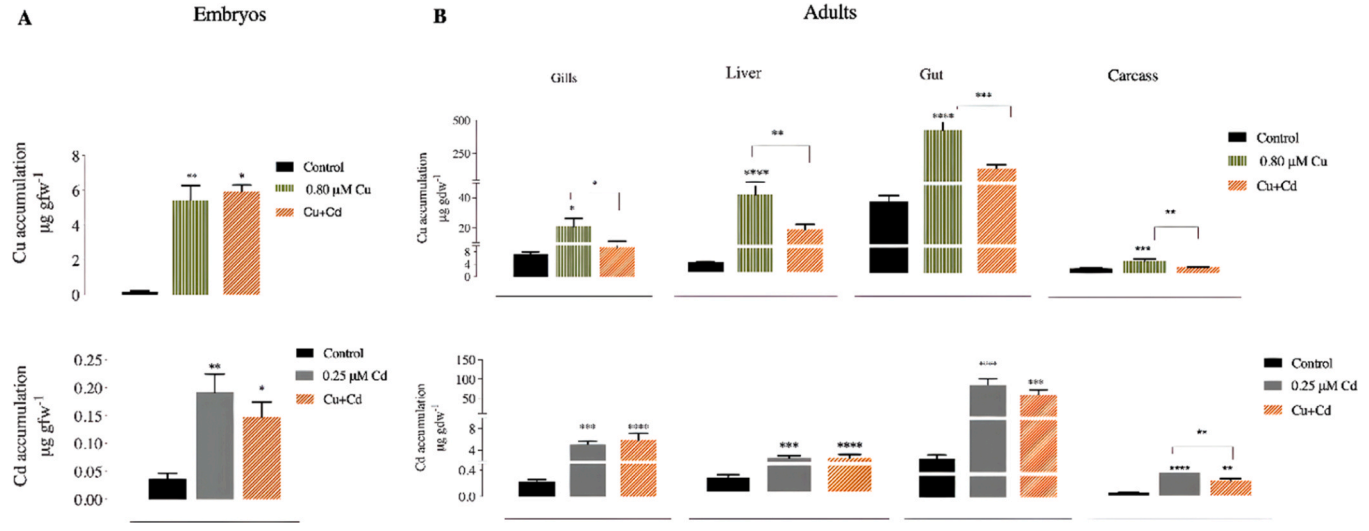

Fig. 4. Metal accumulation. (A) Cu and Cd accumulation in zebrafish embryos 96 hpf exposed to 0.80 μM Cu, 0.25 μM Cd and Cu+Cd. The values are the average of ± standard error of mean (SEM) of minimum ten biological replicates in each exposure group. (B) Cu and Cd concentration in zebrafish adults exposed to 0.80 μM Cu, 0.25 μM Cd and Cu+Cd. The values are the average ± 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. For embryos D'Agostino-Pearson normality test failed, therefore, a non-parametric test (Kruskal-Wallis one way ANOVA) on ranks was performed and Dunn's method was used for multiple comparison. For adults, a parametric test was performed, and Holm-Sidak's multiple comparison test was used to compare the treatment and control group. *p < 0.05, * *p < 0.01, * **p < 0.001, * ** *p < 0.0001.

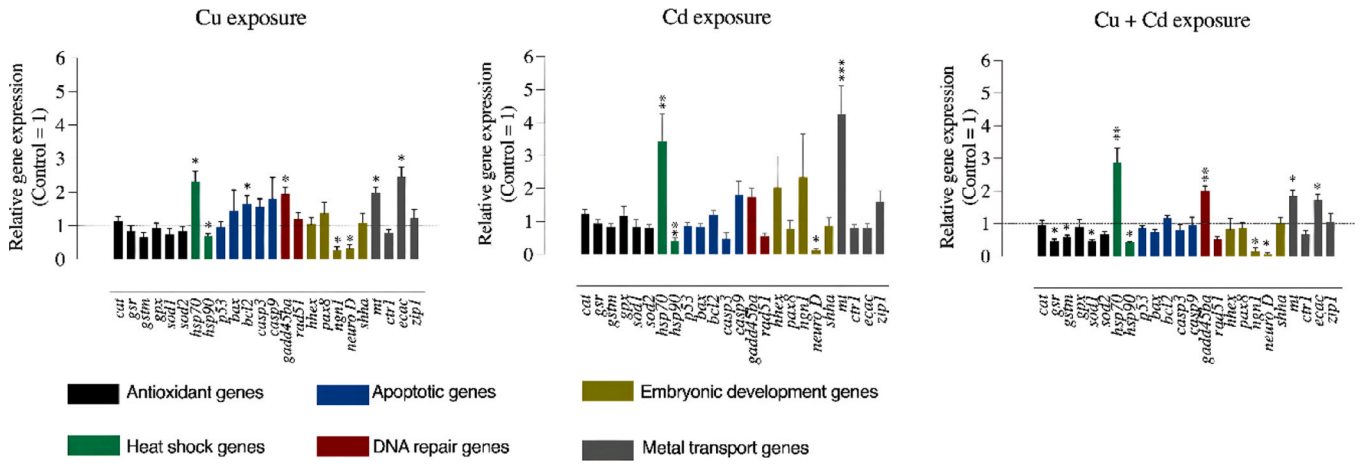

Fig. 5. Gene expression analysis of Zebrafish embryo. Relative gene expression levels of genes representing different classes of genes (antioxidant, apoptosis, development, heat-shock, DNA repair, and metal transport) in zebrafish embryos exposed to 0.80 μM Cu, 0.25 μM Cd and Cu+Cd at 96 hpf. The values 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. A non-parametric test was used to compare the treatment and control groups. *p < 0.05, * *p < 0.01, * **p < 0.001.

direct damage to the chorion, oxidative stress, disruption of ion regulation, and interference with signalling pathways crucial for embryonic development (Jezierska et al., 2009; Martin et al., 2011; Zhang et al., 2018; Taslima et al., 2022). In addition to hatching impairment, several non-lethal effects, including oedema, spinal curvature, impaired eye and head development, and elevated mortality rates were observed, particularly in the Cu+Cd-exposed embryos. This indicates the increased severity associated with combined metal exposure.

Behavioural changes are widely recognised as indicators of neuronal function and neurotoxicity (Johnson et al., 2007; Jin et al., 2015; Jarema et al., 2022). In our study, behavioural changes were also noted, with Cu and Cu+Cd-exposed embryos showing impaired movement and swimming ability, which is indicative of neurotoxic effects. Given the differential responses observed between embryos and adults, future research should focus on the specific mechanisms underlying these effects at different life stages.

Adults exposed to Cu and Cd exhibited fewer overt toxicological signs, and survival rates remained largely unaffected. However, Cu+Cd-exposed adults displayed mild hypoactivity and abdominal swelling. This difference may reflect the more mature physiological and adaptive mechanisms in adults, which could mitigate the immediate impact of these metals. Nonetheless, the mild symptoms observed in Cu+Cd-exposed adults indicate that combined exposure may still induce sublethal effects, potentially through the disruption of homeostatic processes like ion regulation, as shown by changes in metal accumulation patterns (see Fig. 4). These findings are consistent with previous studies that have shown more pronounced effects of metal mixtures on developing stages compared to adults, likely due to developmental stage-specific sensitivities to environmental stressors (Mohammed, 2013; Majid et al., 2022). Embryos, with their rapidly dividing cells and underdeveloped defence systems, are particularly vulnerable to environmental toxicants (Mohammed, 2013). In contrast, adults benefit from more advanced protective mechanisms, such as functional gills and robust detoxification pathways, which help maintain physiological stability under stress (Mohammed, 2013).

Our findings reveal distinct patterns of metal accumulation and their association with the observed toxic effects. The similarity between the effects in embryos exposed to Cu alone and those exposed to the Cu-Cd mixture suggests that Cu is primarily responsible for driving the toxic outcomes, with Cd acting to amplify these effects. This is further supported by our metal accumulation data, which demonstrate that Cd accumulation is suppressed in the presence of Cu (see Fig. 4). Furthermore, the dynamics of metal uptake differed across life stages. In embryos, Cd uptake was reduced when co-exposed to Cu, while in adults, Cu uptake was inhibited by Cd in all examined organs (see Fig. 4). These findings suggest a stage-specific interaction between Cu and Cd, where Cd alters Cu bioavailability and uptake in adult tissues, potentially due to competitive interactions between the metals. The literature supports the existence of inhibitory interactions between Cu and Cd. For instance, Komjarova and Blust (2008) found that Cd inhibited Cu uptake in *Daphnia magna*, and similar interactions were reported by Castaldo et al. (2020) in *Cyprinus carpio*. However, opposite trends have been observed in *Danio rerio* (Komjarova and Blust, 2009a; Komjarova and Bury, 2014). Such interactions often arise even at low concentrations due to the physicochemical similarities between Cu, an essential metal, and Cd, a nonessential one. These similarities can lead to ionic and molecular mimicry, allowing Cd to replace essential metal ions like Zn^{2+}, Cu^{2+}, and Ca^{2+} in metalloenzymes (Bridges and Zalups, 2005; Qu and Zheng, 2024), and affects the function of key biomolecules, including enzymes, proteins, and nucleic acids (Bertin and Averbeck, 2006).

In our study, co-exposure to Cu and Cd significantly decreased Na levels in embryos compared to single-metal exposures (see Supplementary Data, S2), which is consistent with previous reports of Cu-induced Na influx reduction in fish (Delahaut et al., 2020). In adults, organ-specific metal ion patterns were observed, with a marked decrease in Na levels in gill tissue, aligning with findings in common carp (Delahaut et al., 2020). This supports the conclusion that Cu drives toxicity in mixed exposures primarily by disrupting sodium homeostasis through inhibition of Na+ /K+ -ATPase activity (Liao et al., 2023). Copper increases cellular permeability, leading to enhanced sodium loss (Liao et al., 2023). Moreover, co-exposure to Cu and Cd resulted in reduced Ca levels, likely due to their competition for uptake sites. While embryos exhibited decreased calcium levels, liver tissues in adults showed elevated Ca, with transcriptional analysis of the Ca transporter ecac revealing decreased activity, suggesting a compensatory response to high intracellular Ca (Bagur and Hajnóczky, 2017). Additionally, co-exposure led to elevated Mg and zinc levels, with Zn levels linked to the upregulation of the zinc transporter zip1 (see Fig. 6).

Organ-specific analysis revealed the highest concentrations of Cu and Cd in gut tissue across both single and mixture exposure conditions, indicating the role of gut in metal accumulation. Despite higher metal accumulation in single-exposure groups, more severe toxic effects were observed in mixed-exposure groups for both embryos and adults. Although adults accumulated more metals overall, embryos experienced greater toxicity, suggesting that metal accumulation alone does not predict toxicity. Instead, internal bioavailability and the interaction of metals with critical physiological sites play a pivotal role. This reinforces the need to consider both internal and external bioavailability when

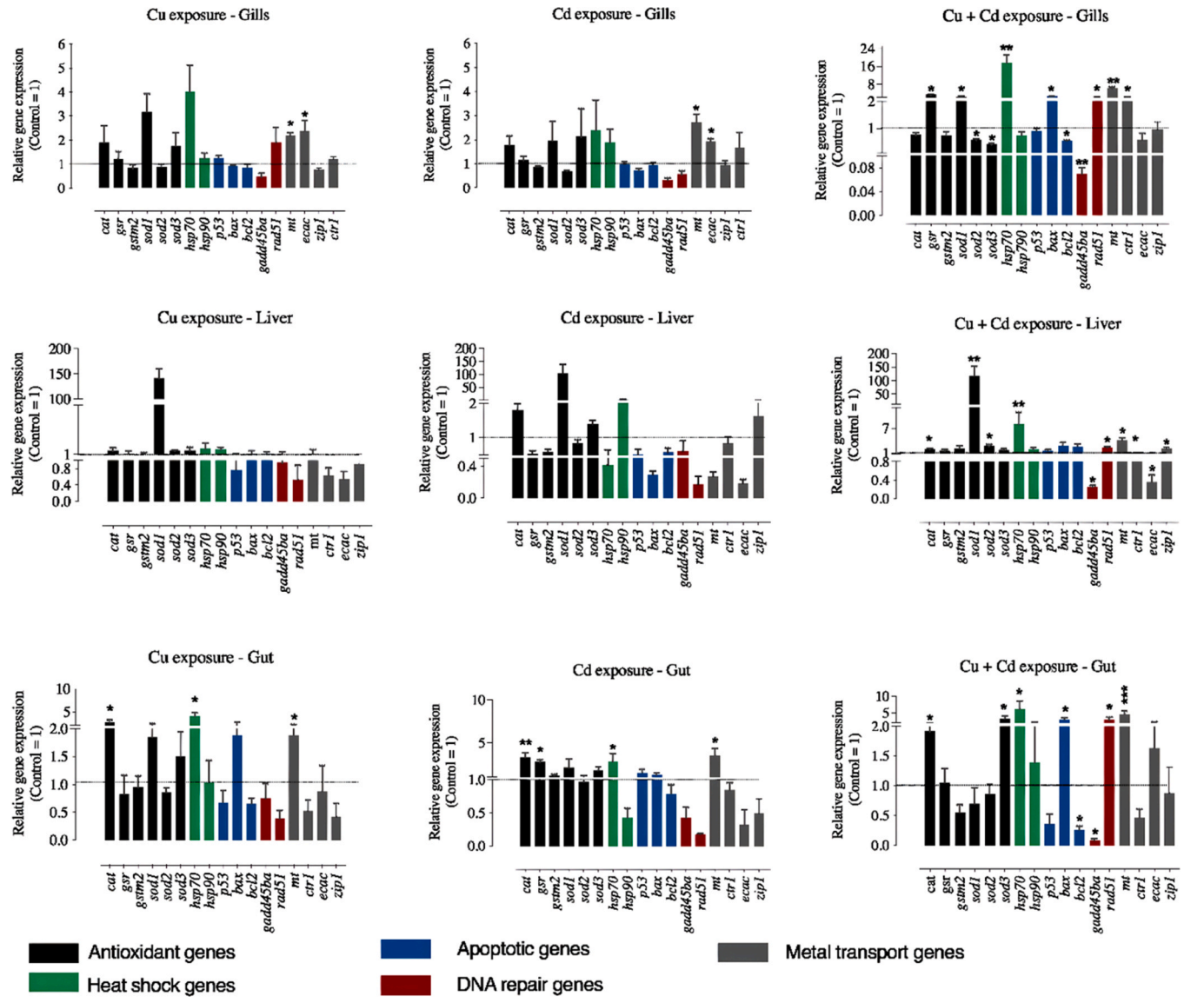

Fig. 6. Gene expression analysis of zebrafish adults. Graphs representing the relative gene expression levels of genes representing different classes of genes (anti-oxidative, apoptosis, metal transport, heat-shock and DNA repair) in gills, liver and gut of adult zebrafish exposed to 0.80 μM Cu, 0.25 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. A non-parametric test based on ranking (Kruskal-Wallis test) was used to compare the treatment and control groups. *p < 0.05, * *p < 0.01, * **p < 0.001.

assessing metal toxicity in aquatic organisms.

To elucidate the mechanism underlying the observed metal-induced developmental effects, we analysed the gene expression patterns associated with (oxidative) stress mechanisms in both embryos and adults (see Figs. 5 and 6). Both Cu and Cd are well-known inducers of oxidative stress, either directly (Cu) or indirectly (Cd), by triggering the production of ROS. Excess ROS can lead to cellular impairment through lipid peroxidation, mitochondrial dysfunction, calcium imbalances, and damage to proteins and DNA (Rensing and Grass, 2003; Valko et al., 2005; Woo et al., 2009; Birben et al., 2012; Collin, 2019). Our data revealed stronger effects on the antioxidative defence system in co-exposed embryos and adults. In embryos, co-exposure resulted in the downregulation of key antioxidant genes (gsr, gstm, and sod1), whereas single-metal exposures did not significantly alter their expression. While the upregulation of antioxidant genes is a common response to oxidative stress, the observed downregulation suggests a more complex regulatory response. One possible explanation is the impairment of protective functions or disruptions in key signaling pathways, such as the Nrf2 (nuclear factor erythroid 2-related factor 2) pathway, which governs antioxidant and detoxification gene regulation (Zhang et al., 2018; Kim et al., 2019; Endo et al., 2020). Although we did not assess Nrf2 activity, the downregulation of antioxidant genes suggests a potential shift in cellular priorities, favouring alternative survival mechanisms over antioxidant defences. Despite the transcriptional downregulation of antioxidant genes, the developmental impairments observed in embryos exposed to either Cu or Cd alone indicate that oxidative stress plays a significant role in toxicity, even when not directly reflected at the gene expression level.

In adults, altered antioxidant gene expression was observed in all three examined organs, suggesting a systemic response to metal toxicity. A consistent stress response across both life stages was the upregulation of hsps and mt, indicative of a shared cellular defence mechanism. Hsp70, a molecular chaperone that responds to various stressors, including oxidative stress-induced protein damage (Mosser et al., 2000; Reeg et al., 2016), was upregulated in all metal-exposed embryo groups and in adults subjected to co-exposure. This highlights its essential role

in mitigating oxidative stress and preventing protein misfolding and aggregation (Ikwegbue et al., 2017; Kurashova et al., 2019). Similarly, mt, a well-established biomarker for Cd exposure, is critical for metal homeostasis, detoxification, and neuroprotection (Vasák and Hasler, 2000; Wang et al., 2014). Its upregulation across both life stages underscores the disruption of metal homeostasis and redox balance, reinforcing its protective role against metal-induced stress. Collectively, these findings suggest that hsp70 and mt play central roles in adaptive responses to metal exposure across developmental stages.

Redox alterations have been widely implicated in adverse neurodevelopmental outcomes (Wells et al., 2009; Salim, 2017), which may explain the neurodevelopmental impairments observed in exposed embryos. This hypothesis is supported by the significant downregulation of neuroD, a key gene involved in neurogenesis and neuronal differentiation (Lee et al., 1995; Miyata et al., 1999; Tutukova et al., 2021). Additionally, metal-induced oxidative stress is known to cause DNA damage (Majid et al., 2022), and our study revealed the activation of DNA damage repair mechanisms in both embryos and adults. However, the differential DNA damage responses between these life stages suggest variations in susceptibility to metal-induced oxidative stress. Specifically, embryos exhibited increased expression of the DNA damage response gene gadd45ba, indicative of increased repair activity, whereas adults showed reduced gadd45ba expression, potentially reflecting a diminished repair capacity or adaptation to sustained damage. Furthermore, rad51, a gene essential for DNA repair, was upregulated in all examined adult organs, indicating a broad activation of repair pathways in response to metal exposure. In contrast, apoptotic gene expression was altered primarily in the gills and gut of adults, two key sites of metal accumulation and elimination. This suggests that metal toxicity may exacerbate cell death in these organs, potentially impairing their detoxification capacity.

Overall, while the underlying mechanisms of metal toxicity (oxidative stress and DNA damage) appear consistent across the two life stages assessed in zebrafish, the gene expression responses varied depending on developmental stage, influencing both the severity and nature of the observed toxic effects. These findings show the importance of considering life-stage-specific responses when evaluating the toxicological impact of environmental contaminants like metals, which may have differential effects on physiological resilience, repair mechanisms, and organ-specific responses.

5. Conclusion

This study investigated the effects and mechanisms of Cu and Cd toxicity, both individually and in combination, in zebrafish embryos and adults. We found that co-exposure to these metals led to pronounced developmental impairments in embryos and functional disruptions in various adult organs. The effects were often absent or milder with single-metal exposure. Oxidative stress emerged as a key toxicity mechanism in both life stages, though distinct defence responses were activated. Our findings challenge the assumption that metal accumulation alone can reliably predict toxicity, highlighting the importance of internal metal dynamics. In addition to its mechanistic insights, our study has several practical implications. The observed metal mixture effects emphasize the need to refine environmental risk assessments, as current regulations often focus on individual metals rather than their combined toxicity. Our findings suggest that water quality guidelines should incorporate mixture-based toxicity evaluations to better protect aquatic life. Additionally, understanding metal-induced oxidative stress responses can inform strategies for mitigating contamination effects in aquaculture and fisheries, where metal pollution poses a threat to fish health. However, a limitation of this study is that mixture exposure concentrations were based on single-metal doses, which may not fully capture the toxicity of equitoxic combinations. Future research should investigate a broader range of exposure concentrations to better understand the complexities of metal mixture toxicity. Additionally, while this study focused on Cu and Cd in zebrafish, further research involving different metal combinations and species is essential to better understand the mixture effects in aquatic ecosystems. Expanding this knowledge base will improve toxicological risk assessments and contribute to more refined environmental safety standards.

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CRediT authorship contribution statement

Sanah Majid: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Karen Smeets**: Writing – review & editing, Supervision, Resources, Conceptualization. **Lucia Vergauwen**: Writing – review & editing, Methodology. **Ali Pilehvar**: Methodology. **Dries Knapen**: Writing – review & editing, Resources. **Ronny Blust**: Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2025.118368.

Data availability

Data will be made available on request.

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