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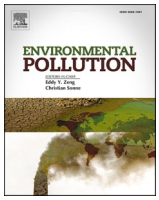

Innovative transgenic zebrafish biosensor for heavy metal detection☆

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ABSTRACT

Heavy metal contamination is an urgent environmental issue that poses a significant threat to human health and the ecosystem. To mitigate the adverse impacts of heavy metal pollution, the aim of this research was to develop genetically engineered zebrafish as biosensors, which offer a promising alternative for detecting heavy metal exposure, specifically Cd^{2+} and Zn^{2+}. A novel heavy metal-sensitive gene construct metallothionine 2 promoter with DsRed reporter gene (*mt2-DsRed2*) was synthesized and integrated into zebrafish embryos using a Tol2 transposon transposase system with the transgenic zebrafish line subjected to biosensing applications for Cd^{2+} and Zn^{2+}. The biosensor showed specific responses with linear correlation heavy metal concentration and DsRed fluorescence signal for Cd^{2+} and Zn^{2+} with ($p < 0.01$) a minimum detection limit of 4 ppb for each metal ion but not for the non-specific metal ion Ni^{2+}, which makes it suitable for laboratory-based heavy metal assessment assays in the low heavy metal concentration ranges of 0–10 ppb. Additionally, the study investigated the toxicity of heavy metals on zebrafish early developmental stages applying a modified version of the OECD Fish Embryo Toxicity (FET-236) test. Accordingly, Cd^{2+}, Zn^{2+}, and Ni^{2+} exhibited no significant toxicity effects on zebrafish embryos within the low dose range of 2–10 ppb confirming the biocompatibility of the transgenic zebrafish biosensor for heavy metal sensing applications. Thus, the developed transgenic zebrafish line can accurately sense heavy metals Cd^{2+} and Zn^{2+} within the low dose range, making it a promising alternative laboratory assay for environmental monitoring and risk assessment.

1. Introduction

Various procedures are available for detecting heavy metal concentrations in biological and environmental samples. Conventional methods include atomic absorption spectroscopy (AAS) and atomic emission spectroscopy (AES) (Kneip, T. J. et al., 1973; Yeung et al., 2017), which are highly accurate, they are associated with high costs and labour-intensive sample preparation and detection procedures. In response to these limitations, biosensors have been developed as an alternative solution, that convert biological parameters into easily detectable signals (Mehrotra, 2016). Fluorescent biosensors are one of the most widely used, possessing fluorescent reporter genes that have been fused to promoters of relevant genes and emit fluorescence in response to exposure to specific analytes. Common fluorescent reporter genes used in these biosensors include Green Fluorescent Protein (GFP), enhanced Green Fluorescent Protein (eGFP), and *Discosoma* sp. Red Fluorescent Protein (DsRed) (Carvan, M. J. et al., 2006). These reporter genes are fused with promoters that are sensitive to heavy metals, allowing activation and expression of the reporter gene in response to heavy metal exposure. Genetically engineered biosensors, especially those based on zebrafish, have emerged as highly effective tools for environmental monitoring due to their sensitivity, cost-effectiveness, and biological relevance in toxicity detection (Bambino & Chu, 2016).

Zebrafish (*Danio rerio*) are aquatic vertebrates with significant homology to their terrestrial counterparts. While zebrafish have a well-established history record in developmental and regenerative biology,

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their utility has grown exponentially with the onset of modern genetics. Genetically engineered zebrafish biosensors have been developed to detect environmental estrogens, which are endocrine-disrupting chemicals (EDCs) associated with decreases in semen quality/sperm count, increased incidence of breast cancer and testicular germ cell cancer, and urogenital tract malformation (Sumpter and Johnson, 2005).Zebrafish-based biosensors for heavy metal detection are promising tools for environmental monitoring and risk assessment due to their high sensitivity and specificity. Such biosensors are classically based on the induction of heat shock proteins (HSPs) as a response to heavy metal exposure, which results in the activation of HSP promoter elements. HSPs are constitutively expressed in most cell types, making them an ideal biomarker for heavy metal exposure (Li et al., 2022; Blechinger et al., 2002). Recent studies have shown that imaging techniques such as Full Field X-Ray Fluorescence (FF-XRF) can be used to detect and map the accumulation of heavy metals on zebrafish. In one study, zebrafish were exposed to three different contaminants over a period of days, and the results obtained using the 2D-THCOBRA (Thick Gas Electron Multiplier with Coplanar Readout Anode) imaging system showed distinct accumulation patterns for each metal (Kim et al., 2022). Genetically engineered microbial biosensors have been widely explored for environmental monitoring due to their ability to detect specific contaminants. However, they face several practical limitations, including reduced stability under variable environmental conditions, high maintenance requirements, and a lack of adaptability to real-world scenarios (Gavrilaș et al., 2022; Herath et al., 2023). Organic molecules serve as effective fluorescent sensors for detecting highly toxic heavy metal cations in potable water (Iftikhar et al., 2023). Additionally, their responses can be influenced by non-target substances, limiting their specificity. These challenges make it difficult for microbial biosensors to serve as reliable and scalable tools for detecting heavy metals in complex environmental matrices. Advancements in genetically engineered zebrafish as biosensors offer a promising alternative, as they provide a biologically relevant platform for assessing toxic substances. Zebrafish models are particularly suitable for environmental applications due to their ability to mimic vertebrate-specific pathways and their sensitive responses to heavy metals (Bera et al., 2014). However, existing biosensors—both microbial and vertebrate-based—often lack the sensitivity required to detect low concentrations of specific heavy metals, such as Cd^{2+} and Zn^{2+} (Pawar et al., 2016).

Another approach to biosensor development involves the reconstruction of metal-sensitive vectors using zebrafish *metallothionein* (*mt*) promoter and fluorescent reporter genes such as eGFP. In a recent study, zebrafish has been proposed as one of the ideal tools for toxicology studies, making them a promising candidate for the development of heavy metal biosensors (Pawar et al., 2016). Overall, zebrafish-based biosensors offer a promising avenue for heavy metal detection in aquatic environments (Pawar et al., 2016). In spite of ongoing efforts, additional research is required to enhance biosensor design and broaden its suitability for detecting various heavy metals under diverse environmental conditions. While fluorescent biosensors offer promise for environmental monitoring, current systems often lack the sensitivity and specificity required to detect trace levels of heavy metals like Cd^{2+} and Zn^{2+} in complex environmental matrices. Traditional chemical analysis methods may not accurately reflect the bioavailable fraction of these metals, which is the portion that is biologically active and can pose risks to organisms (Zhu et al., 2023). To address these limitations, there is a need for advanced biosensors that can detect low-level metal exposure and provide accurate assessments of bioavailability. By incorporating advanced signal processing techniques, such as image analysis, and developing biosensors with improved specificity, it is possible to create more sensitive and reliable monitoring tools. Additionally, integrating these biosensors with other analytical techniques, such as MP-AES, can provide a comprehensive understanding of metal exposure and toxicity.

The use of zebrafish cell lines and whole organisms as biosensing tools has been explored, yet a significant gap remains in the literature regarding vertebrate-based biosensing for heavy metals. Current methods lack a bioavailable, cost-effective, and less labour-intensive laboratory tool for detecting heavy metals (Wan et al., 2009). Despite the identification of several heavy metal-responsive genes, no standardized laboratory assays utilize these genes in vertebrate models. To address this gap, our study represents one of the first attempts to develop a standardized laboratory assay for detecting heavy metals using zebrafish cell lines and whole organisms as biosensors. By leveraging the unique bio sensitivity of zebrafish genes and promoters to specific heavy metals, this approach provides a bioavailable, cost-effective, and less labour-intensive alternative to traditional methods. Furthermore, the assay will be standardized through detailed Standard Operating Procedures (SOPs) to ensure reproducibility and reliability in laboratory settings. In this study, a newly designed heavy metal sensitive gene construct (*mt2-DsRed2*) was synthesized and integrated into zebrafish genome using a gene integration system The transgenic zebrafish line was confirmed for transgenicity in F_1 generation with eGFP reporter expression and subjected to biosensing applications for $Cd^{2+}$and Zn^{2+} using *DsRed2* fluorescent reporter gene. Further, a comprehensive laboratory based Cd^{2+} and Zn^{2+} fluorescent detection assay was developed and calibrated (with fluorescent microscopy and digital image processing tools) utilizing embryos of transgenic fish line to quantify the Cd^{2+} and Zn^{2+} in a provided sample. Additionally, the study investigated the adverse effect of heavy metals (Cd^{2+}, Zn^{2+} and Ni^{2+}) on zebrafish early development stages using a modified version of the OECD Fish Embryo Toxicity (OECD FET-236 assay) test to identify the compatibility of our biosensor in heavy metal detection applications. Taken together, the transgenic zebrafish line developed in this study can accurately sense specific heavy metals within the low dose range, making it a promising tool for environmental monitoring and risk assessment in laboratory samples.

2. Material and methodology

2.1. Generation of biosensor construct

The full-length genomic DNA sequence of the zebrafish *metallothionein2* gene (*mt2*) was retrieved from National Centre for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/nucleotide/) and the region corresponding to the *mt2* gene promoter was annotated (Accession No: EU847278.1). The Metal Response Elements (MREs), AP1 region, GC boxes and TATA box on the promoter sequence were identified. The Tol2 transposon (pT2AL200R150G)/ transposase helper plasmid (pCS-zT2TP) system was from Kawakami Lab, National institute of Genetics, Japan (https://kawakami.lab.nig.ac.jp) The gene sequence of *DsRed2* was retrieved from the NCBI database and fused with pre-annotated *mt2* promoter sequence using SnapGene viewer tool (https://www.snapgene.com). This way chemically synthesized *and* incorporated into the pT2AL200R150G donor plasmid at Genscript via De novo gene synthesis techniques and validated via GenSmart™ Codon Optimization tool.

For future PCR validations of the vector system and the heavy metal sensitive gene construct, two separate primer sets were designed and validated using OligoAnalyzer (https://sg.idtdna.com/pages/tools/oligoanalyzer). A primary set of primers were designed to amplify the mt2-DsRed2 region in the transposon donor plasmid and the secondary set of primers were designed to be used to amplify the transposase gene in the helper plasmid (Supp. Tables 1,2,3).

2.2. Zebrafish rearing and breeding

Wildtype zebrafish were maintained in a 1400-tank Techniplast zebrafish aquatics system with custom life support system (Techniplast, Italy) at Deakin University Animal Phenomics Facility, Waurn Ponds, Victoria, Australia. Adult zebrafish were maintained in 3 L tanks at 27 °C with continuous flow of water and a photoperiod of 12 h light: 12h dark.

The chemical parameters of the aquarium water including pH (7 ± 0.5) and electric conductivity (470–530 μs/m) were monitored daily and nitrates (<0.009gm/L), nitrite (8–12 mg/L) and ammonia levels (<0.05 mg/L) monitored weekly. For breeding and to obtain zebrafish embryos for microinjection experiments, two females and one male zebrafish were transferred to 3 L breeding tanks containing a 2 mm mesh insert and transparent barrier separating the male and female overnight. Next morning the barrier was removed and spawned embryos were collected from the tanks using mech strainer and they were transferred to a 10 cm Petri dish containing 25 mL E3 media with 0.3 mg/L methylene blue. Any dead or unfertilized eggs observed in black colour were removed from the dish by using suction from a transfer pipette and the remaining fertilized eggs were immediately transferred to another petri plate incubated at 28 °C and prepared for subsequent microinjection rounds. All animals used in this study were approved for use by the Deakin University Animal Ethics Committee following the Australian Code for the Care and Use of Animals for Scientific Purposes (AEC number: G26-2019).

2.3. Zebrafish transgenesis

Transgenic fish production was conducted at PC2 certified laboratories in Deakin University, Australia, following the guidelines of the Australian government's Office of the Gene Technology Regulator (OGTR).

In-vitro transcription of the pCS-zT2TP vector was performed using the mMESSAGE mMACHINE® Kit with SP6 RNA polymerase, after the vector was linearized with Not1 restriction digestion. The transposon vector (pT2AL200R150G) and *in-vitro* transcribed mRNA (from Transposes pCSZT2P) were mixed in a 1:1 ratio and loaded into micropipette needles were prepared using a micropipette needle puller with specific parameters (heat–365, pull–45, velocity–80, time–150, pressure–500)

Healthy wildtype zebrafish embryos at the one-cell stage (30 min.–45 min post fertilization) were injected with 2–5 nL of injection solution. After injection, embryos were placed in a Petri dish with fresh E3 media containing 0.3 mg/L of methylene blue for 15 min to recover before transferring them to a 28 °C incubator.

At 72 hpf (hour post-fertilization) larvae were transferred into a 6-well plate, anesthetized with E3 media containing 0.1 mg/mL of benzocaine and imaged using Olympus Cell Sens Standard software with an Olympus MVX10 fluorescence microscope (Olympus, model: MVX10 with a LH100HG and a U-MGFPA/XL GFP mirror filter attached to a DP74 camera for photography). Constant exposure settings were used throughout the imaging of experimental eggs and larvae.

The fluorescent confirmed F_0 generation transgenic larvae were grown up to maturity for 12 weeks and subjected to breed with wildtype fish The 24 hpf and 72 hpf progeny larvae were subjected to fluorescent microscopy to identify in F_1 generation.

Genomic DNA was extracted from both transgenic and wildtype individuals with Qiagen DNAeasy blood and tissue kit following the manufacturer's protocol). Successful integration of DNA construct was confirmed by PCR using primer pair listed in Supp.Ta ble. 1 **which was designed to amplify the** *mt2-DsRed2* region. PCR mixture was prepared as indicated in the Supp. Table 2. **The PCR conditions were set as indicated in the** Supp. Table 3. **PCR products of both transgenic and wildtype were run in a 1 % agarose gel with 100 bp DNA Ladder.**

2.4. Fluorescence analysis for heavy metal detection

The heavy metal solutions were prepared for subsequent zebrafish biosensor calibration assays. Initially, the 100 ppm stock solutions of Cd^{2+}, Zn^{2+} and Ni^{2+} were prepared by using $CdCl_2$, $Zn(CH_3COO)_2$, $Ni(NO_3)_2.6H_2O$ (Sigma-Aldrich) respectively and dissolving them in ddH_2O in new and autoclaved 15 mL Falcon tubes. The heavy metal standards of 0.1 ppm, 0.5 ppm, 1.0 ppm, 2.0 ppm, 3.0 ppm, 4.0 ppm and 5.0 ppm prepared from each metal by following a serial dilution method (Godshaw J. et al., 2017) and stored at room temperature for subsequent treatments. All the storage, handling, treatments, discarding of heavy metal contaminated samples and environmental releasing regulation were carried out by strictly following the standard Material Safety Data Sheet (MSDS) protocols as described by Sigma-Aldrich.

F0 transgenic zebrafish were bred to generate F1 embryos for heavy metal testing. Transgenicity of F1 embryos was confirmed using fluorescence microscopy before metal exposure. The heavy metal series were freshly prepared on the day of treatments for biosensor signal calibration treatment assays from previously prepared and stored Cd^{2+}, Zn^{2+} and Ni^{2+} standard solutions. The 0.1 ppm of Cd^{2+}, Zn^{2+} and Ni^{2+} of standard vials were retrieved from the fridge and utilized in preparing 2, 4, 6 8 and 10 ppb dilution series in new and autoclaved 1.5 mL Eppendorf tubes for separate Cd^{2+}, Zn^{2+} and Ni^{2+} treatment series.

Healthy 72 hpf F1 zebrafish larvae and free of structural deformities, were used for heavy metal treatments. Ten larvae were added to each well of 6-well plates containing 3 ml of E3 media. The larvae were exposed to varying concentrations (0, 2, 4, 6, 8, and 10 ppb) of Cd^{2+}, Pb^{2+}, and Ni^{2+} solutions for 24 h at 28 °C (Fig. 1). All experiments were performed in triplicate within a PC-2 certified zebrafish research facility.

After a 24-h incubation with different heavy metal concentrations, larvae were anesthetized with E3 media containing 0.1 mg/ml benzocaine for fluorescence microscopy analysis. Following washes with egg water, larvae were positioned for imaging using a standardized protocol on an Olympus MVX10 fluorescence microscope equipped with a DsRed mirror filter. Consistent exposure settings ensured comparable data collection. ImageJ software (https://imagej.nih.gov/ij/) was then used to quantify the metal-induced response. Red fluorescence images, captured in TIFF format, were imported into ImageJ. Here, a binary conversion process transformed the images into black and white, allowing for the measurement of pixel intensity (a measure of fluorescence) for each larva. This analysis, performed on nine larvae per dose, provided the data necessary to plot graphs correlating relative fluorescence intensity with the administered heavy metal concentration.

2.5. Zebrafish embryo toxicity test

The zebrafish embryo toxicity experiments were conducted with transgenic zebrafish using a modified version of the OECD FET test. Ten 24 hpf MT2:DsRed transgenic zebrafish embryos, confirmed by fluorescence microscopy in the F1 generation, were added to each well of a 6-well plate containing aquatic test media., zebrafish aquatics water (pH ± 0.5 and electric conductivity 470–530 μs/mL) or E3 media (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, pH 7.2). Aquatic test media was removed from the wells and 5 mL of aquatic test media containing increasing doses of Cd^{2+}, Zn^{2+} and Ni^{2+} (2, 4, 6, 8, 10 ppb) were added to the wells. The zebrafish embryos in 6-well plates were cultured in a Labec environmental incubator at 28 °C, 80% relative humidity and photoperiod of 12 light:12 dark throughout the experiment. Zebrafish embryo hatch and the mortality were evaluated and recorded at 24, 48, 54, 72, 80 hpf intervals. The number of embryos hatched vs those that were not hatched at each time point expressed as the percentage hatch rate. Likewise, the total number of living embryos vs dead embryos in each exposure dose and at each time point are reported as percentage survival rate. The heart rate of zebrafish embryos (beats per minute) at 72 hpf was recorded in the control treatment and each Cd^{2+}, Zn^{2+} and Ni^{2+} (2, 4, 6, 8, 10 ppb) dose using direct microscopic observation and a stopwatch. The photographs of zebrafish embryos exposed to the control (H_2O) and varying dose of Cd^{2+}, Zn^{2+} and Ni^{2+} (2, 4, 6, 8, 10 ppb) were captured at 72 hpf using a dissecting microscope and a camera, images were then analyzed for zebrafish embryo developmental abnormalities including, no structural deformity (NSD), yolk sac edema (YSE), pericardial edema (PE), and spinal curvature (SC). All developmental abnormalities were recorded and the percentage of each abnormality per well was calculated.

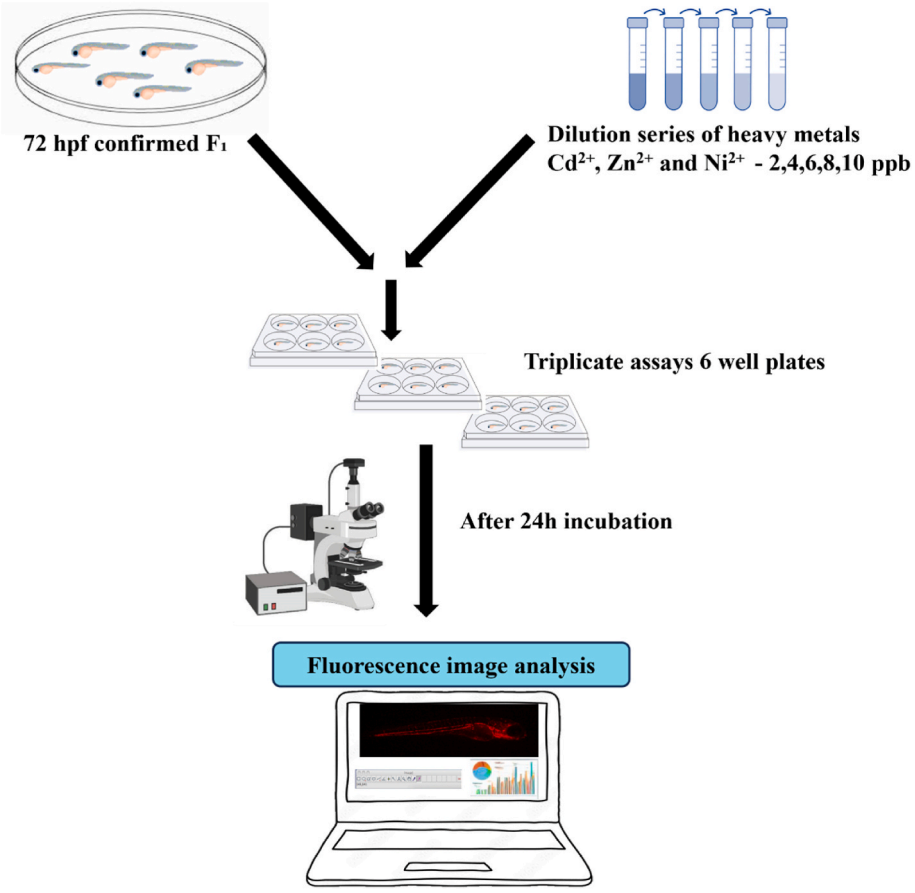

Fig. 1. Experimental Setup for Heavy Metal Detection Assay. Zebrafish F1 generation larvae at 72 hpf and free of structural deformities, ten larvae per well, in triplicate, were cultured in 6-well plates with, Each well received 3 mL of control (0 ppb) or varying concentrations (2, 4, 6, 8, and 10 ppb) of Cd^{2+}, Pb^{2+}, and Ni^{2+} solutions and plates were then incubated at 28 °C for 24 h. Subsequently, larvae were examined under fluorescent microscopy.

2.6. Statistical analysis

Raw data were collated and analyzed using GraphPad Prism 8.2.1. Assumptions of homogeneity of variance and normality were checked, and two-way Analysis of Variance (ANOVA) was used to compare dose-dependent differences in the response variables (survival rate, hatch rate, heart rate, and developmental abnormalities) compared to the control ddH_2O dose within each Cd^{2+}, Zn^{2+} and Ni^{2+} treatment experiment. On the response variables (Cd^{2+}, Pb^{2+} and Zn^{2+} concentration doses) compared to the control non-treated transgenic fish. If significant differences ($p < 0.05$) were found, a Dunnet's multiple comparison test was carried out to identify these significant differences. To elaborate the significance difference between adjacent heavy metal doses, Turkey's multiple comparison test was applied.

3. Results

3.1. Generation of transgenic zebrafish

A heavy metal sensitive DNA construct was designed. This included the *mt2* promoter region of that contained "TATAA box" and "GC box" which are typical features of a gene promoter and 10 Metal Responsive Elements (MREs) that are distinct elements contained by metal responsive and metal homeostasis family genes fused to the reporter

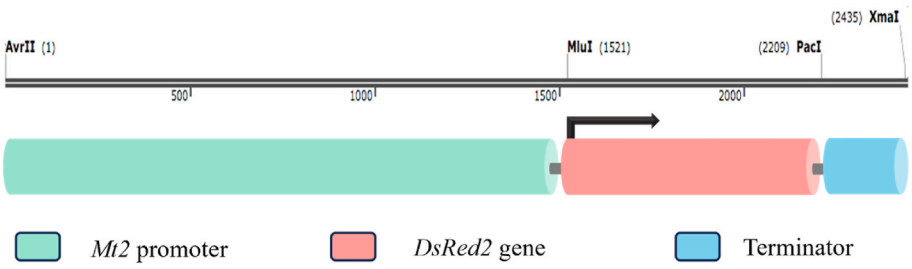

Fig. 2. Structure of the Heavy Metal-Sensitive DNA Circuit. The computationally designed heavy metal-sensitive DNA construct, encompassing a total length of 2435 base pairs (bp). This comprised the *mt2* promoter region, marked by the light green colour cylinder, that spans 1521 bp, the DsRed2 coding region of the reporter gene denoted by the light pink colour cylinder, measures 688 bp and a terminator sequence denoted in blue featuring a stop codon and the SV40 polyadenylation signal. Restriction sites, including *Avr*II at the beginning, *Mlu*I between the *mt2* promoter and DsRed2 coding regions, *Pac*I at the initiation of the terminator sequence, and *Xma*I at the conclusion of the DNA construct are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

gene (*DsRed2* coding region) (Fig. 2). This was cloned into a Tol2 integration vector, with the resulting constructed injected into zebrafish embryos along with transposase and a terminator sequence containing a stop codon with the SV 40 polyadenylation signal downstream.

Potential transgenics identified by the presence of green fluorescent signal in F_1 generation of zebrafish larvae with, significant green colour signals were observed in the head and along the latter body of fish embryonic stage in ubiquitous manner (Fig. 3). Transgenics were confirmed by PCR genomic DNA extracted from F_1 generation transgenic larvae. by observing a 431 bp long PCR band in agarose gel electrophoresis (**Supp. Fig. 1**).

3.2. Response to heavy metals

Zebrafish larvae in F_1 generation at the age of 72 hpf were exposed to different doses of Cd^{2+}, Zn^{2+} and Ni^{2+} and observed for red colour fluorescence. Transgenic larvae displayed red colour fluorescent signals in dose dependent manner (Fig. 4A). As compared to untreated control fish, Cd^{2+} treated fish showed significant increase signals from 4 ppb and above. The difference between 4 ppb compared to 6 ppb, difference between 6 ppb compared to 8 ppb and difference between 8 ppb compared to 10 ppb were significant as well. In Zn^{2+} treatment, 72 hpf F1 transgenic larvae displayed dose-dependent red fluorescence (Fig. 4B). Significant signals emerged from 4 ppb Zn^{2+} treatment onwards. The difference between 4 ppb and 6 ppb treatment was insignificant but differences between 6 ppb to 8 ppb and 8 ppb–10 ppb have been significantly different. When 72 hpf old F_1 generation transgenic larvae were treated with Ni^{2+}, none of the treatments within the treatment range of 0–10 ppb exhibited significant red fluorescent responses as compared untreated transgenic fish (Fig. 4C).

3.3. Biocompatibility of transgenic zebrafish in heavy metal detection applications

A few other key biological parameters were also examined of transgenic zebrafish embryos exposed to different doses of Cd^{2+}, Zn^{2+}, and Ni^{2+}. Across the doses examined (0–10 ppb), larvae exposed to all three metal ions showed no significant differences from controls in terms of hatching (Fig. 5I) survival rates (Fig. 5 II) heart rates (Fig. 5III). Structural deformities, including Pericardial Edema (PE), Yolk Sack Edema (YSE), and Spinal Curvature (SC), (Fig. 6-I). This comprehensive assessment of biocompatibility provides insights into the effects of heavy metals on zebrafish development, hatch rates, survival rates, heart rates, and structural deformities if there was any defect to be observed (Fig. 6-II).

4. Discussion

Zebrafish has been identified as a suitable biological system to develop biosensors for detecting heavy metals in contaminated water samples due to its potential applicability in aquatic environments and its ability to capture bioavailable heavy metals (Iqbal et al., 2023). Multiple attempts have been made to develop biosensors for detecting heavy metals using genetically engineered zebrafish strains with metallothionein promoters coupled with eGFP to detect Zn^{2+} and Cd^{2+} within their regulatory limits (Iu et al., 2016) and MT-Ia1 coupled with DsRed2

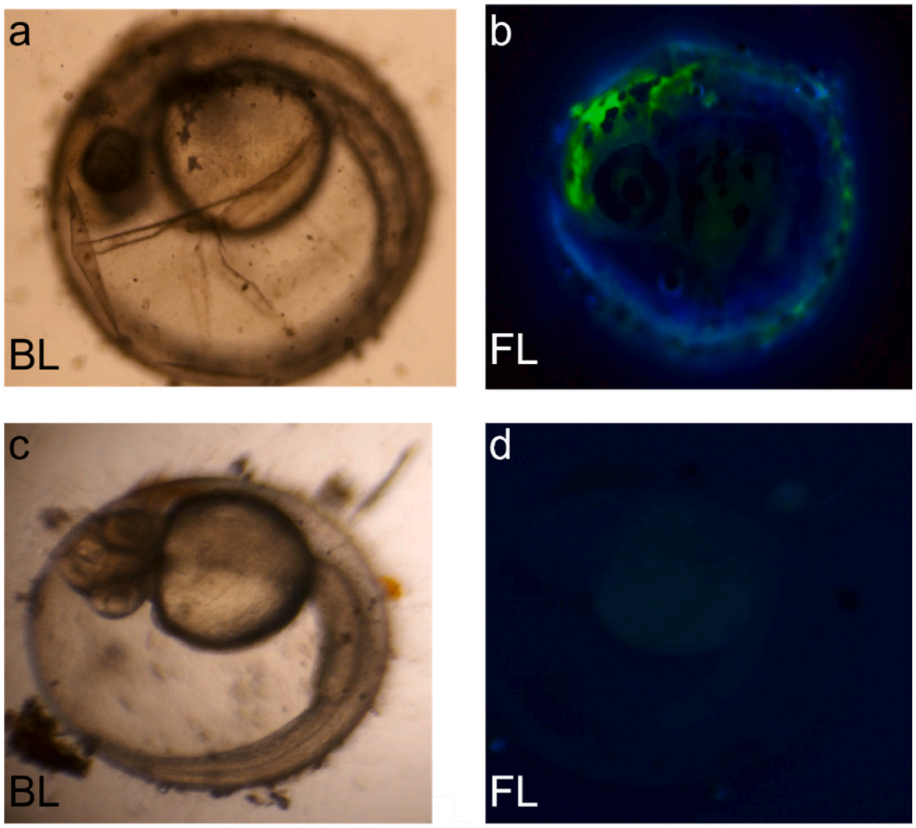

Fig. 3. Fluorescence microscopy visualization of eGFP expression for transgene integration confirmation in zebrafish.
a and b: These images compare 24 hpf embryos. The top left image (a) shows a transgenic embryo under brightfield microscopy, while the top right image (b) shows the same embryo with green fluorescence under the FITC filter.
c and d: These images depicted as controls, showing a wildlife-type embryo under brightfield bottom left image (c) and bottom right image FITC filter (d) at 24 hpf. No green fluorescence is present in the wildtype embryo. (Magnification: × 40; BL – Bright-Field, FL Fluorescence). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

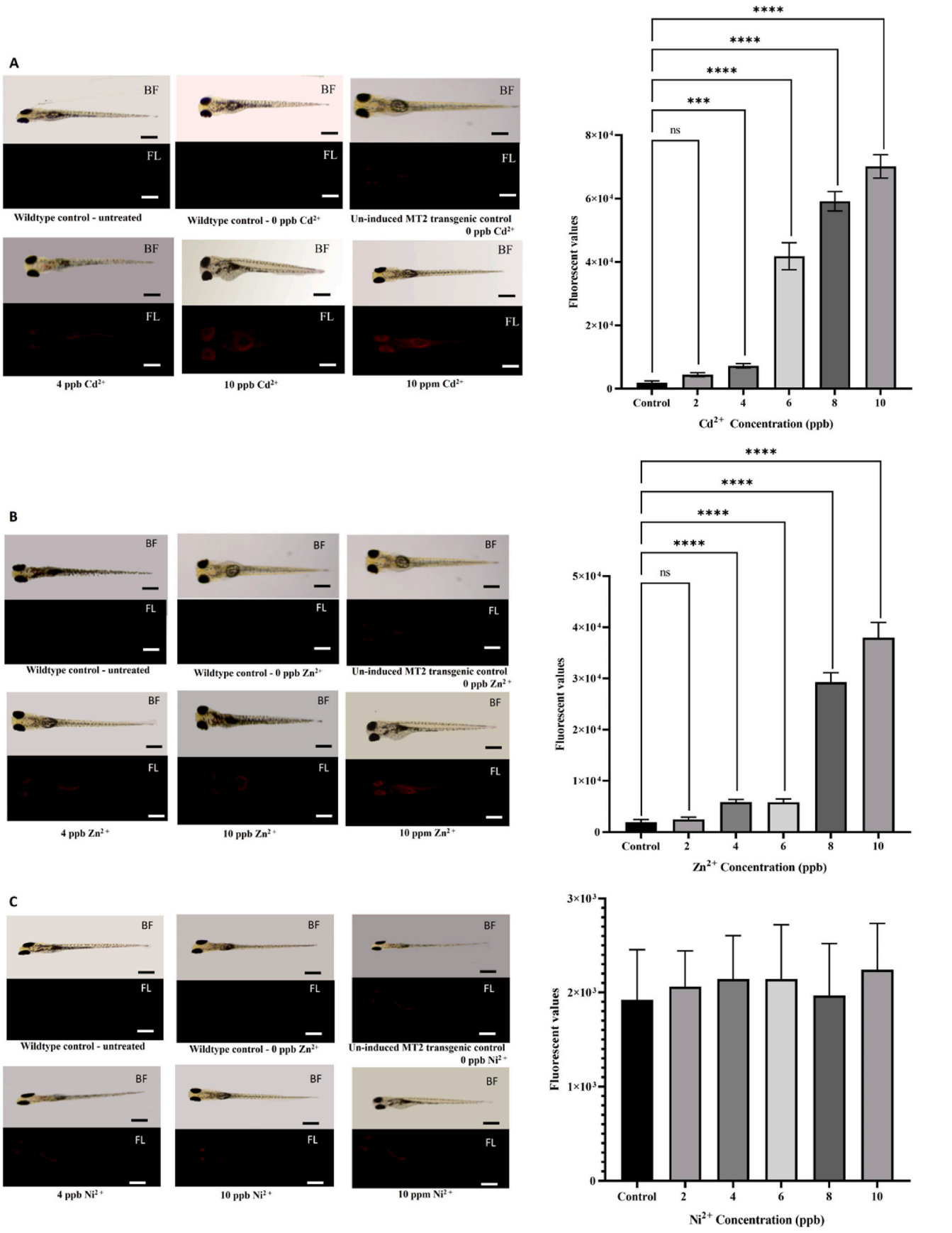

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Fig. 4. Analysis of Heavy Metal-Induced DsRed Fluorescent Signals in F1 Transgenic Zebrafish larvae. Fluorescent (FL) microscopic images of wild-type and transgenic (left hand panel) 72 hpf zebrafish larvae Bright-field (BL) and corresponding incubated with the indicated heavy metals. Corresponding plots depict the relationship between heavy metal concentrations and red fluorescent values, (right hand panel). Each treatment point represents the average mean value of red fluorescent signal intensities normalized to background fluorescence from un-induced controls calculated from ten biological replicates across three experimental replicates (totalling 30 fish). Each treatment indicates the mean ± SEM of nine individual fish. Statistical significance is denoted as follows: ns (not significant), ***$p < 0.01$, ****$p < 0.001$. A. Cd^{2+} Treatment B. Zn^{2+} Treatment C. Ni^{2+} Treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

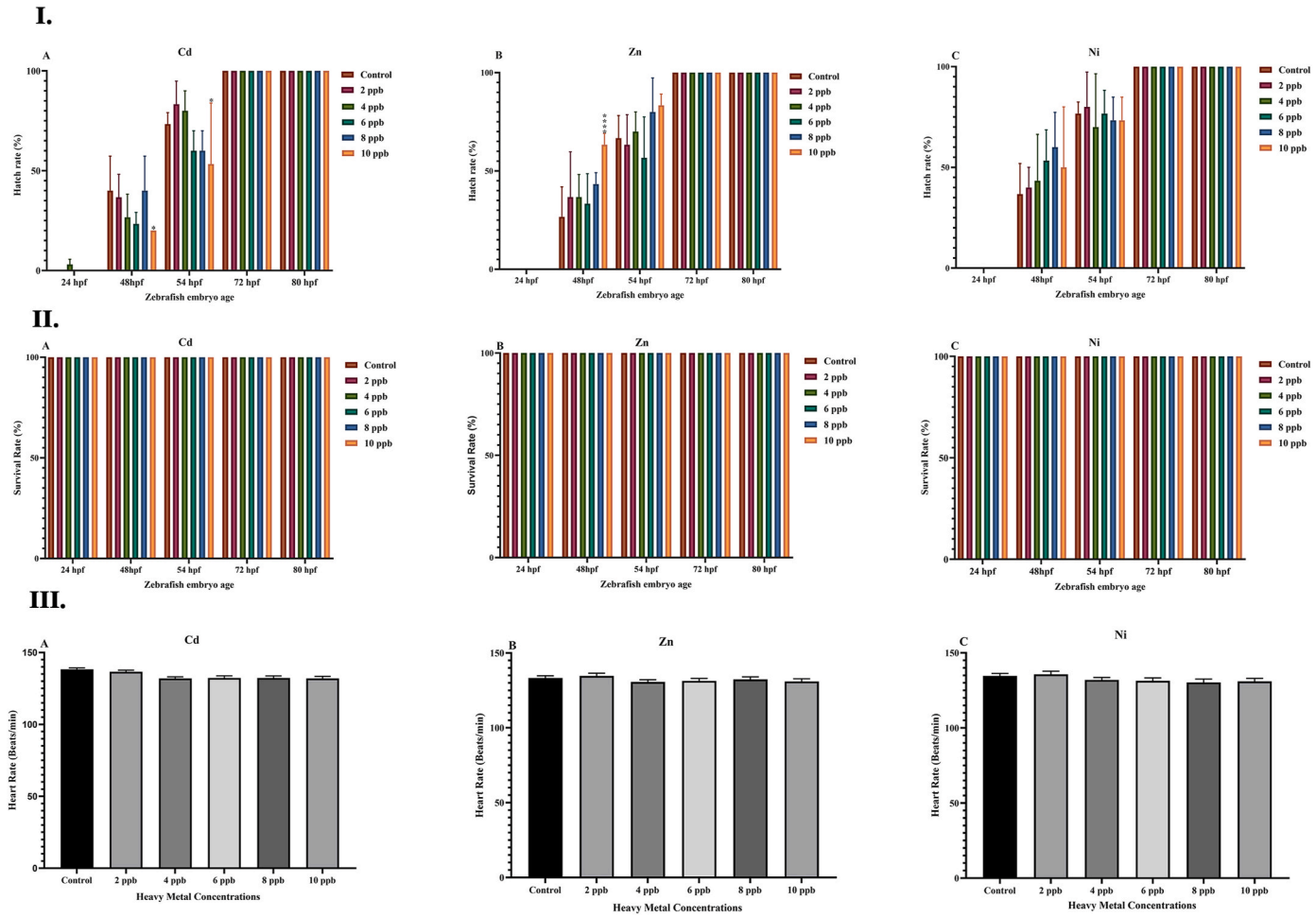

Fig. 5. (I) Hatching Rates of Transgenic Zebrafish Embryos Exposed to Increasing Heavy Metal Concentrations. Hatching rates (%) of transgenic zebrafish embryos at 24, 48, 54, 72, and 80 hpf following exposure to escalating concentrations of heavy metals (2, 4, 6, 8, 10 ppb). (A) Cd^{2+} (B) Zn^{2+} (C) Ni^{2+}
(II) Survival Rate of Transgenic Zebrafish Embryos Exposed to Increasing Heavy Metal Concentrations. Survival rates (%) of transgenic zebrafish embryos at 24, 48, 54, 72, and 80 hpf after exposure to escalating concentrations of 2, 4, 6, 8, 10 ppb. (A) Cd^{2+} (B) Zn^{2+} (C) Ni^{2+}
(III) Heart Rate (beats/min) of 72 hpf Transgenic Zebrafish Embryos Exposed to Incremental Heavy Metal Concentrations. Heart rate (beats/min) of 72 hpf transgenic zebrafish embryos following exposure to escalating concentrations of 0, 2, 4, 6, 8, 10 ppb. (A) Cd^{2+} (B) Zn^{2+} (C) Ni^{2+}.

to detect Cd^{2+} with a minimum detection limit of 0.5 mg/L (Pawar, N. et al., 2016). By integrating this genetic construct into zebrafish embryos, a transgenic zebrafish line with high sensitivity to aquatic zinc (Zn) and cadmium (Cd) was obtained (Wang et al., 2016). Additionally, zebrafish-based biosensors have been developed for detecting other environmental pollutants and a wide range of medical applications. For instance, specific promoters coupled with luciferase and fluorescent have been used in a Zebrafish biosensor to monitor numerous polycyclic hydrocarbons and halogenated coplanar molecules such as TCDD and polychlorinated biphenyls (Carvan et al., 2000). Moreover, researchers have used zebrafish-based biosensing to study the effects of smoke-based particles from different automobiles on animal development. In medical applications, zebrafish has been utilized as a biosensor to study complex neurological phenomena of neurotransmission and neuromodulation (Leopold A. V. et al., 2019).

Despite these studies, there is currently no standardized laboratory assay or field detection system utilizing genetically engineered zebrafish-based biosensors that effectively meets the requirements of a reliable and appropriate method for ecotoxicological assessment. Thus, our study mainly focuses on developing a Cd^{2+} and Zn^{2+} sensitive transgenic zebrafish biosensor paying close attention to calibration and biocompatibility validation aspects, and to propose laboratory based heavy metal detection assay plan. Accordingly, to develop a biosensor for targeted heavy metals, the *mt2* gene was selected as the core component of the genetic construct due to its selectivity towards Cd^{2+} and Zn^{2+} as shown in previous studies (Andrews, 1990). When targeted heavy metals are present, the *mt2* promoter becomes induced, activating gene expression. This, in turn, triggers the transcription of the red fluorescent protein corresponding gene segment linked to the mt2 promoter, resulting in the production of *DsRed2* mRNA and red fluorescent

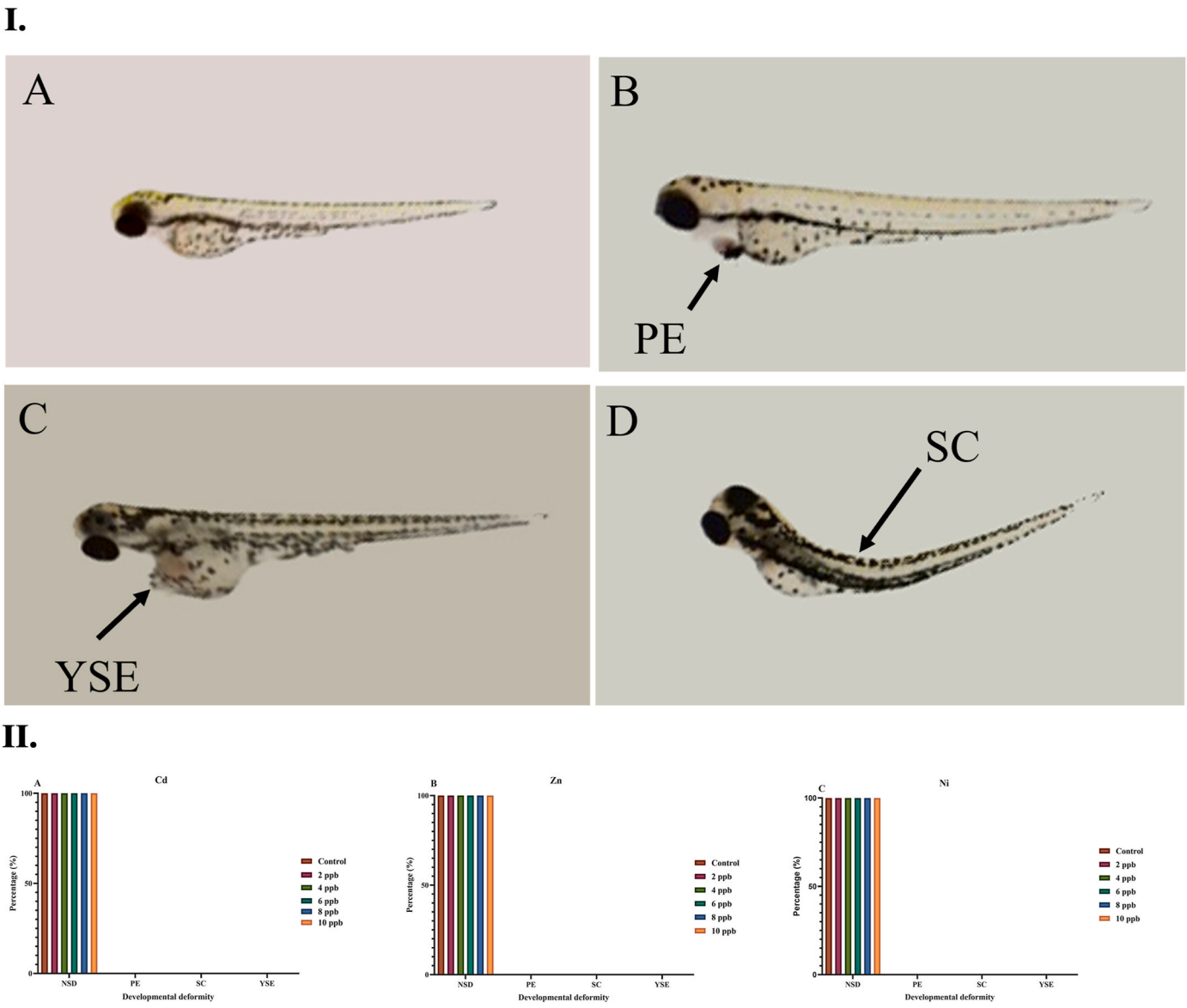

Fig. 6. Microscopic Examination of Structural Deformities in 96 hpf Zebrafish Larvae (i.) Structural Deformities Examined: (A.) No Structural Deformities (NSD) (B.) Pericardial Edema (PE)(C.) Yolk Sac Edema (YSE) (D.) Spinal Curvature (SC) Scale bar = 3 mm in each image. (ii.) Plots Between Developmental Deformities and Percentage (%): (A.) Transgenic zebrafish larvae of 96 hpf exposed to Cd^{2+} in aquatic water (0, 2, 4, 6, 8, 10 ppb). (B.) Transgenic zebrafish larvae of 96 hpf exposed to Zn^{2+} in aquatic water (0, 2, 4, 6, 8, 10 ppb). (C.) Transgenic zebrafish larvae of 96 hpf exposed to Ni^{2+} in aquatic water (0, 2, 4, 6, 8, 10 ppb). Asterisk ($* = p \leq 0.05$) denotes the concentration at which heart rate is significantly different from control zebrafish. Values represent mean ± SEM (n = 4–6).

protein. We confirmed the activation of the MT2 promoter through a gene expression analysis of the DsRed reporter. Our results demonstrated that Cd^{2+} and Zn^{2+}, unlike Ni^{2+} and Cu^{2+}, elicited a concentration-dependent upregulation of DsRed fluorescence at ppb levels (see Supp. Fig. 2). The incorporation of restriction enzyme sites at the beginning, between the *mt2* promoter region and *DsRed2* gene sequence, *DsRed* gene sequence and terminator sequence, and at the end of the gene construct allows for chemical alterations downstream if necessary. Additionally, a terminator sequence is attached to the end of the heavy metal detecting genetic construct to ensure accurate termination of *DsRed* mRNA transcription. The genetic construct developed in this study has the potential to be a highly sensitive and selective biosensor for detecting targeted heavy metals in aquatic environments.

We utilized the Tol2 helper/donor vector system (pCS-zT2TP and pT2AL200R150G) to incorporate the chemically synthesized heavy metal sensitive genetic construct into the zebrafish genome. Tol2 is the preferred transposon system for zebrafish-based experiments due to its high efficiency and applicability in insertional mutagenesis (Urasaki, A. et al., 2008). While piggyBac is another gene integration kit that shows great potential in transposon-based reprogramming of induced-pluripotent stem cells for regenerative medicine, the Tol2 system was selected as it is more efficient and applicable in zebrafish studies. The helper plasmid codes for the enzyme that excises the DNA elements within the Tol2 upstream and downstream ends, while the transposon donor plasmid can transpose insert sizes of up to 11.5 kb without reducing the germline transmission frequency (Urasaki, A. et al., 2006). The gene cassette used in this study is within the Tol2 left and right ends, which span a region of 4643 bp, thereby falling within the successful transposable insert size range.

As established by previous studies on transgenic zebrafish, extrachromosomal transgenes are maintained through many rounds of DNA replication during the early phases of embryonic development. While some transgenes are randomly integrated into the host genome at a later stage, others are degraded, resulting in the production of mosaic F_0

transgenic fish (Clark, K. J. et al., 2011). In order to establish a founder line of transgenic fish, F_0 transgenic individuals are crossed with wild-type fish. To confirm successful integration of the gene circuit into the zebrafish genome in our study, a green fluorescent protein marker was incorporated into the construct (eGFP marker). High levels of green fluorescence were observed in the F_1 generation of embryos and larvae, confirming the successful integration of the *mt2-Dsred2* construct into the zebrafish genome. This was achieved by growing the microinjected F_0 generation fish to maturity and crossing them with wildtypes to obtain stable transgenics. Notably, no green signals were observed in wildtype fish, indicating that the transgene had been successfully integrated into the genome of transgenic fish only (Clark, K. J. et al., 2011).

When 72 hpf larvae were exposed to Cd^{2+}, Zn^{2+} and Ni^{2+}, the fluorescent intensity results showed that Cd^{2+} and Zn^{2+}, which are specific to the *mt2* gene promoter, exhibited significant red fluorescent signals in a dose-dependent manner, while the genetic circuit did not produce detectable signals for the non-specific Ni^{2+} metal ion. This observation indicated that our gene circuit selectively responds to targeted metal ions based on the specific metal-responsive features of the *mt2* gene promoter. The minimum detection limit for both Cd^{2+} and Zn^{2+} with the current microscopic setup was 4 ppb. Furthermore, the intensity of the signals generated for Cd^{2+} was higher compared to those for Zn^{2+}. The responsive nature of the *mt2* promoter-based gene circuit to both Cd^{2+} and Zn^{2+} is justifiable based on the close resemblance of the chemical properties of the two metal ions. The higher intensity of Cd^{2+} signals may be due to its higher compatibility and binding capacity with the MTF-1 transcription factor than Zn^{2+}.

This present study shows the potential of utilizing genetically engineered zebrafish as biosensors for heavy metal detection. Our study further highlights the ability of a transgenic zebrafish strain with an *mt2*-based gene circuit to selectively detect bioavailable levels of Cd^{2+} and Zn^{2+}. Although a live organism (zebrafish) was used as the detection component of the biosensor, the downstream analysis and signal calibration were digitized using fluorescent microscopy and digital image processing techniques with ImageJ software. This digital processing makes our biosensor a promising candidate for developing rapid on-site heavy metal detection devices with minimal labour required. In addition, since the gene circuit is germline integrated in fish, large numbers of zebrafish larvae can be produced for multiple detection assays. To further improve the sensitivity of the biosensor, advanced studies can be conducted to optimize the transgenic fish design. For example, in-crossing two transgenic F_1 generation heterozygous fish to obtain F_2 generation stable homozygous transgenic fish with two copies of the genetic construct of interest could enhance the sensitivity of the fluorescent signals. This would enable naked-eye detection of the fluorescent signals using low-cost image processing strategies, which would eliminate the need for expensive microscopic image capturing setups. Overall, our technique shows great potential in replacing existing laboratory-based, high-cost heavy metal detection protocols in the future.

The latter study aimed to address the knowledge gap regarding the toxic effects of Cd^{2+}, Zn^{2+}, and Ni^{2+} on a novel heavy metal-sensitive transgenic zebrafish line before its application in actual heavy metal detection. To this end, we tested the Mt2-DsRED transgenic zebrafish against Cd^{2+}, Zn^{2+}, and Ni^{2+} at doses ranging from 0 to 10 ppb within 96 hpf. The choice of this time points and the dose range was intended to ensure that the normal physiological processes of zebrafish embryos and larvae were not compromised, as such, the zebrafish biosensor could function accurately. To evaluate the toxicological effects, we conducted a modified version of the OECD-FET 236 assay, which involved examining hatch rates, survival rates, heart rates, and developmental deformities resulting from Cd^{2+}, Zn^{2+}, and Ni^{2+} exposure.

The hatching process in zebrafish is a critical developmental milestone initiated when the embryo outgrows its oxygen supply obtained through diffusion from the egg envelopes and perivitelline fluid (Muller et al., 2015). This process involves chorion softening facilitated by the zebrafish hatching enzyme (ZHE1), a proteolytic enzyme (Schoots et al., 1983). Heavy metal exposure at this stage can disrupt the hatching enzyme and inhibit normal hatching (Korwin-Kossakowski, 2012). Hatch rates were evaluated using low doses of Cd^{2+}, Zn^{2+}, and Ni^{2+} (0–10 ppb) to ensure minimal impact on zebrafish embryos' normal physiological processes (Westerfield, 2000). These low doses did not significantly affect hatch rates. In previous studies (Aldavood S. J. et al., 2020; Yang et al., 2021), it has been demonstrated to have adverse effects on hatch rates at higher metal concentrations.

Survival rates of zebrafish embryos were monitored at various time points. At low doses (0–10 ppb), embryos showed similar survival rates to non-treated controls. In previous studies, survival rates were observed to be impacted by the higher doses of Cd^{2+}, Zn^{2+}, and Ni^{2+} exposure (Wu, S.M. et al., 2013; Jin, Y. et al., 2015; Yang et al., 2021; Aldavood et al., 2020).

Cardiovascular development in vertebrates, occurring during the early stages of embryogenesis, is sensitive to environmental pollutants such as heavy metals, leading to cardiovascular abnormalities (Lawson and Weinstein, 2002b; Herbert & Stainier, 2011; Mizell et al., 1996). This study measured heart rates in response to heavy metal exposure. Accordingly, heart rates at 72 hpf remained unaffected by Cd^{2+}, Zn^{2+}, and Ni^{2+} at both low heavy metal doses. However, previous studies reported significant changes in heart rates following metal exposure (Yang et al., 2021; Mitovic N. et al., 2021).

The study also assessed structural deformities, including pericardial edemas, yolk sack edemas, and spinal curvatures, in response to heavy metal exposure. Low doses did not result in deformities in transgenic larvae tested. Previous research has indicated the susceptibility of fish embryos to osmotic imbalances caused by heavy metals (Kiener et al., 2008; Hill et al., 2004). The chorion layer plays a pivotal role in shielding embryos from toxicants while maintaining osmotic balance in fish embryos (Wu & Zhou, 2012). The disruption of the circulatory system and osmoregulation may lead to deformities in fish embryos (Incardona and Scholz, 2016). Histochemical staining revealed the importance of tight junction proteins like TJP3/Zo-3 in osmoregulation during early development (Kiener et al., 2008).

Accordingly, our study investigated the sensitivity of transgenic zebrafish embryos to Cd^{2+}, Zn^{2+}, and Ni^{2+} exposure during early developmental stages, using concentrations ranging from 0 to 10 ppb. At these lower doses (0–10 ppb), no significant effects on hatch rates, survival rates, or heart rates were observed. Therefore, our pre-designed zebrafish biosensor can be used for low dose detection of Cd^{2+} and Zn^{2+} without inducing any toxic effects on zebrafish embryos during early development.

Taken together, this study presents a novel biosensor for the detection of heavy metals using zebrafish as the host biological organism (Supp. Table 4). The biosensor was created by microinjecting a chemically synthesized heavy metal sensitive gene construct, designed with the promoter sequence of the *mt2* gene and *DsRed2* fluorescent reporter gene coding sequence, into zebrafish embryos. The study highlights the potential of transgenic zebrafish embryos for the detection of Cd^{2+} and Zn^{2+} heavy metals. Consequently, transgenic zebrafish embryos can serve as a promising tool for laboratory-scale, quantitative assays to assess heavy metal contamination.

5. Conclusion

This study successfully developed a novel and sensitive biosensor for low-dose heavy metal detection using genetically engineered zebrafish. This design enables specific and dose-dependent red fluorescent responses to Cd^{2+} and Zn^{2+} with a minimum detection limit of 4 ppb. Notably, the transgenic zebrafish exhibited no significant toxicological effects on hatch rates, survival rates, heart rates, or developmental deformities when exposed to low doses (0–10 ppb) of Cd^{2+}, Zn^{2+}, and Ni^{2+}, confirming its biocompatibility for heavy metal sensing applications. These findings hold significant promise for the future of heavy

metal monitoring and environmental risk assessment. Future research directions could focus on expanding the detection repertoire of the biosensor to encompass a broader range of heavy metals. Additionally, development efforts can be directed towards creating a more robust biosensor system suitable for field-based heavy metal monitoring applications.

CRediT authorship contribution statement

H.M.L.P.B. Herath: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Visualization, Data curation, Writing – original draft, Writing – review & editing. **Rohini M. de Silva:** Supervision, Methodology, Resources, Project administration, Funding acquisition, Writing – original draft. **R.S. Dassanayake:** Supervision, Project administration, Data curation, Writing – review & editing. **Y.I.N. S. Gunawardene:** Methodology, Data curation, Writing – original draft, Supervision. **J.R.P. Jayasingha:** Methodology, Data curation, Formal analysis, Software, Visualization, Writing – original draft. **A. Schultz:** Supervision, Methodology, Writing – review & editing. **C. Liongue:** Supervision, Methodology, Writing – review & editing. **A.C. Ward:** Methodology, Resources, Writing – review & editing. **L.O.B. Afonso:** Methodology, Project administration, Funding acquisition, Writing – original draft, Supervision. **K.M. Nalin de Silva:** Supervision, Resources, Project administration, Methodology, Funding acquisition, Writing – original draft.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Prof. Rohini M. de Silva reports financial support, administrative support, and equipment, drugs, or supplies were provided by National Research Council of Sri Lanka. If there are other authors, they 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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2024.125547.

Data availability

All data has been disclosed

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