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Determination of the Toxicity Cause by Trace Metals on Zebrafish (*Danio rerio*) Embryo

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Abstract

Water quality deterioration due to inorganic and organic pollutant is a serious issue and the presence of toxic trace metals cause a serious threat to the aquatic ecosystem. Fish embryos have gained interest in risk assessment because of their high sensitivity to pollutants and the ecological relevance. This study investigated the acute toxicity effect of trace metals Arsenic (As), Cadmium (Cd), Mercury (Hg), Lead (Pb), Copper (Cu) and Zinc (Zn) on zebrafish (*Danio rerio*) embryo. Embryos were exposed to ten different concentrations of individual trace metals and lethality rate was recorded at 24, 48, 72 and 96 hours based on the coagulation of fertilized egg, lack of somite formation, lack of detachment of the tail and lack of heart beat. The results indicated a significant difference between the control and trace metal treated embryo ($P < 0.05$) and higher mortality rate along the increase of trace metal concentration. Along with the increase of exposure time for Cu, Zn and As, the mortality rate became slower. Sub-lethal and teratogenic deformities such as growth retardation, lack of tail development, lack of eye lens (placode), yolk sac edema, pericardial edema, hemorrhages, shrinkage of chorion and scoliosis were observed in most of the trace metal treated embryos. The results showed the toxic effects to aquatic biota due to trace metals emphasizing the usefulness of zebrafish embryo model for integrated biological hazard assessment.

INTRODUCTION

Water quality deterioration is a major problem in most countries, due to organic and inorganic contaminants. Among the aquatic toxicants, trace metals cause serious impacts on the aquatic ecosystem and organisms. Metals are a natural component of the aquatic ecosystem (Goel, 2006) and trace metals such as Copper (Cu), Zinc (Zn) and Chromium (Cr) are important for the metabolic and other biological activities of aquatic organisms. Mercury (Hg), Lead (Pb) and Cadmium(Cd) are biologically non-essential metals that can be toxic to biota even at a very low concentration (Mamboya, 2007). When some essential trace metals (Cu, Zn, and Cr) present at high concentration, exceeding the maximum limit, can lead to toxicity (Ebrahimi and Taherianfard, 2011;

Annabi *et al*., 2013a). Trace metal pollution in many aquatic environments has increased due to anthropogenic activities, mostly by the industrial effluent releasing, domestic waste and urban run-off, garbage dumping, dumping of automobile waste and mining activities (Goel, 2006; Gharedaashi *et al*., 2013). High accumulation of trace metal in both biotic and abiotic components causes serious health consequences. Thus, assessment of their toxicity has become an important component of water pollution monitoring. Furthermore, in Sri Lanka, Chronic Kidney Disease of unknown origin is a serious issue since the 1990s and one of the causes for this disease is due to chronic exposure of heavy metals at low levels (Bandara *et al*., 2008; Diyabalanage *et al*., 2016 and Jayalal *et al*., 2019). Hence determining the trace metal contamination of water and the toxic effects have become a timely necessity. In this study, zebrafish model was used as a bio-indicator to determine the effects of trace metal pollution.

Fish are the non-mammalian vertebrates used in toxicity testing (Lammer and Braunbeck, 2006). Several fish species are recommended for standard testing of chemicals. Among them, zebrafish (*Danio rerio*) have special characteristics, which advance its use as a model organism. The zebrafish is a tropical freshwater fish, a member of the family Cyprinidae. It is distributed throughout South and Southeast Asia (Lawrence, 2007). Traditionally it was used in molecular genetics, but now it is focusing on several chemical testing and pathological processes as it is having several specialties; small in size, all major organs present within five days post-fertilization, short generation time (3-4 months), produces 300-400 eggs every two weeks, easily available non-adhesive eggs obtained from abundant spawning, translucent embryos, rapid embryonic development and lots of genome resources available.

Toxicity tests are desirable in water quality evaluation because chemical and physical tests alone are not sufficient to assess the potential effect on aquatic biota (Rice *et al.*, 2012). Toxicity tests will reveal the organisms' sensitivity to a particular toxicant that would help us to determine the permissible limit of a toxicant in an ecosystem (Nekoubin *et al*., 2012). Since the implementation of the Animal Welfare Guideline 86/609/EC in 1986, the development and validation of alternatives to animal testing are strongly promoted by the European Union (EU) institutions (Council Directive, 1986). As alternative fish embryos are used instead of adult fish in present toxicology studies. Now in most of the toxicity studies zebrafish embryos are used as an alternative model for the fish acute toxicity and to determine the toxicity of pollutants. Therefore, this study was focused on the determination of the acute toxicity of Cu, Zn, Cd, As, Pb and Hg that produce a lethal effect on zebrafish embryos during short-term exposure.

METHODOLOGY

Place and Time

The experiment was carried out at the Zebrafish Research Facility of the Department of Animal Science, Faculty of Animal Science and Export Agriculture, Uva Wellassa University, Badulla, Sri Lanka.

Research Materials

To determine the toxic effects of trace metals, zebrafish embryos were used as an alternative for laboratory animal testing. Adult, wild-type zebrafish were reared at the Zebrafish Research Facility used for breeding and obtained the embryos before the blastula phase (~ 16 cell stage) at 1.5 - 2 hours post-fertilization.

Research Design

Acute Cu, Zn, Pb, As, Cd and Hg toxicity experiments were performed for a period of four days (until hatching) using zebrafish embryos. Initially, a range-finding test was carried out in the nominal

concentrations (1000, 100, 10, 1 and 0.1 mgL^{-1}) of the selected trace metals, and the zebrafish embryotoxicity was observed until 96 hours post-exposure. Ten eggs per concentration were exposed in a flat bottom 96 well plate and each treatment were repeated three times. Deionized water was used as a negative control and 15% (Hassan *et al*., 2008) ethanol was used as the positive control (Figure 1).

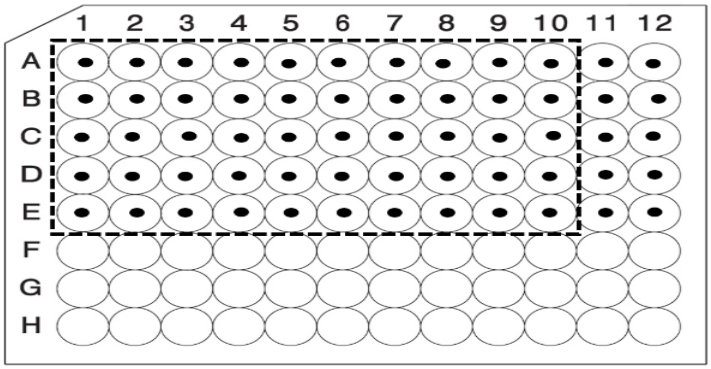

Figure 1. Schematic diagram of the range finding assay of a trace metal. A-E: Five range finding concentrations (1000, 100, 10, 1 and 0.1 mgL^{-1}). 1-12: Zebrafish embryos; single embryo per well is indicated by a black dot. Embryos at columns number 11 and 12 were exposed to negative control (deionized water) and positive control (15% ethanol) respectively. Three 96 well plates with similar arrangements were used as replicates for each trace metal.

After range finding test, ten concentrations were tested against 16-cell stage zebrafish embryos for six individual trace metals. Deionized water was used as the negative control and as the internal plate control. As a positive control concentration of 15% ethanol was used. Twenty embryos (n=20) per treatment (trace metal concentration) with three replicates, were randomly selected and transferred into the respective concentrations and controls. The experiment design of exposing embryos for ten concentrations of a trace metal as illustrated in Figure 2.

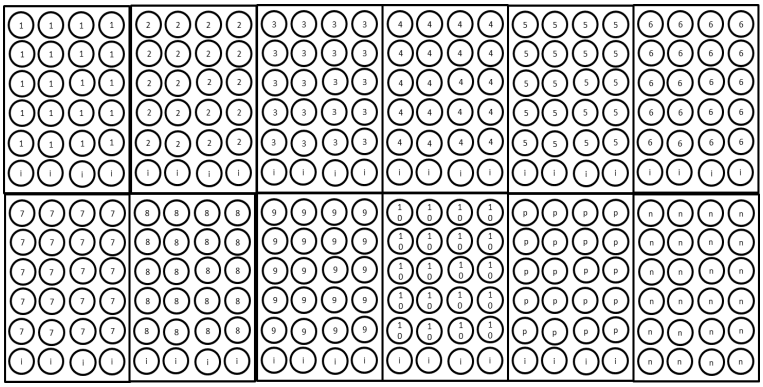

Figure 2. Schematic diagram of the experiment, 1-10 ; test concentrations, i ; internal plate control (deionized water), p ; positive control (15 % ethanol), n ; negative control (deionized water). Single 16-cell stage zebrafish embryo was placed in each well of the 24 well plates.

Work Procedures

Apparently healthy, adult (~ six months old) male and female zebrafish were selected for breeding at a male : female ratio of 2:1. The spawning tank was prepared by placing spawn traps on the previous day to prevent cannibalism.

Four males and two female fish were placed on a four-liter glass tank prepared for breeding the day before collecting eggs. The embryos were collected using a small pipette with a wide opening (Figure 3) 30 minutes after the sun rise.

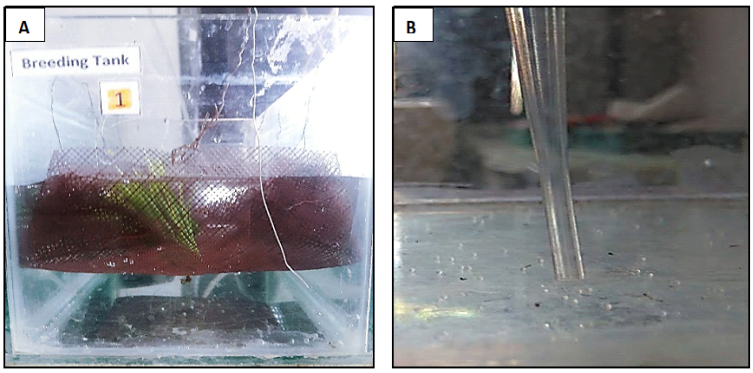

Figure 3. Preparation of spawning tank for breeding (A) and the collection of zebrafish embryos using pipette (B).

The stock solution of 1000 ppm of selected metal (Cu, Zn, Pb, Cd, As and Hg) were prepared a day before the test by dissolving Copper sulfate ($CuSO_4.5H_2O$), Zinc sulfate ($ZnSO_4.\ 7H_2O$), Lead nitrate ($Pb(NO_3)_2$, Arsenic pentoxide (As_2O_5), Cadmium chloride ($CdCl_2.\ ½\ H_2O$) and Mercury chloride ($HgCl_2$) in deionized water. The working treatment solution was prepared daily by serial dilution from the stock solution.

Based on the results of the range finding test, ten test concentrations of Cu (0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 and 10.0 mgL^{-1}), Zn (2.0, 4.0, 8.0, 16.0, 32.0,64.0, 125.0,250.0, 500.0 and 1000.0 mgL^{-1}), Pb (2.0, 4.0, 8.0, 16.0, 32.0,64.0, 125.0,250.0, 500.0 and 1000.0 mgL^{-1}), As (2.0, 4.0, 8.0, 16.0, 32.0,64.0,125.0,250.0, 500.0 and 1000.0 mgL^{-1}), Cd (0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 and 10.0 mgL^{-1}) and Hg (0.002, 0.004, 0.008, 0.016, 0.032, 0.064, 0.125, 0.250, 0.500 and 1.0 mgL^{-1}) were prepared by diluting the stock solution in deionized water. Deionized water was used as the negative control and the internal plate control. As a positive control concentration of 15% ethanol were used. Fertilized zebrafish embryos were immersed in the test solutions before cleavage, at ~ 16 cell-stage.

The fertilized embryos were distributed randomly on 24 well plates which were pre-conditioned for 24 hours and refilled with 2 mL per well freshly prepared test solutions as shown in figure 2 and covered with self-adhesive foil to incubate at 26 ± 1 °C.

The mortality of the fish eggs was recorded at 24, 48, 72 and 96 hours of exposure using a stereo-microscope by considering four indicators of lethality namely, coagulation of fertilized egg, lack of somite formation, lack of detachment of the tail and lack of heartbeat (OECD, 2013). The dead or coagulated eggs were removed immediately.

Data Analysis

The acute toxic effect of each metal on the zebrafish embryo was determined by Probit Analysis LC_{50} determination method. Confidential limits were calculated with a 95% confidence interval (CI) and data analysis was performed by using statistical software SPSS 15.

RESULTS AND DISCUSSION

The present study was carried out to find the acute toxicity effects of trace metals on zebrafish embryos. According to the analysis in the range finding test, the minimum concentration that can cause 100% mortality was recorded. Based on the results obtained, ranges for As, Pb and Zn were below 1000.0 mgL^{-1}, for Cd and Cu, it was less than 10.0 mgL^{-1} and for Hg,

less than 1.0 mgL^{-1} . The relationship between the metal concentration and mortality rate of each trace metal was recorded based on the four apical endpoints. It showed that the mortality rate was increasing with the increase of trace metal concentration.

Acute toxicity of As, Cd, Hg, Pb, Cu and Zn indicated that mortality was directly proportional to the concentration of the trace metals. The Probit analysis revealed that there was a significant difference between the trace metals (As, Cd, Pb, Hg, Cu, and Zn) and the control group ($P < 0.05$). At 24 hours post-fertilization (hpf), none of the embryos exposed to 15% ethanol were alive and 100% of embryos exposed to negative and internal controls and the lowest concentration of As (2 mgL^{-1}) and Pb (2 mgL^{-1}) were alive. After 48 hpf one or more embryo deaths were observed in all treatments except the negative and internal controls. In general, the hatchability of the zebrafish embryo occurs after the 72 hpf, and in this study, trace metals affected the hatchability of some embryos. In negative and internal controls more than 90% of the embryos were hatched at 72 hpf, whereas in the metal treated groups low hatchability was observed at higher concentrations (supplementary data tables S1-S6).

Early hatching was also noted due to the weakening or disruption of chorionic membrane integrity by trace metals (Weis, 2014). Chorion, which is a semi-permeable protective layer in fish embryos unable to prevent trace metal penetration due to the formation of pores, altering the permeability of the chorionic membrane. However, chorion act as a barrier to prevent Cd transfer to the developing embryo (Shazili and Pascoe, 1986). In zebrafish, it was reported that 61% of total Cd was retained by the chorion (Annabi *et al*., 2013b) which is also shown from the findings of this study.

Median Lethal Concentration

The median lethal concentration (LC_{50}) of each trace metal at 24 hpf, 48 hpf, 72 hpf, and 96 hpf are summarized in Table 1. According to the analysis of LC_{50}, Hg is highly toxic to the zebrafish embryo followed by Cu, Cd, Zn, As and Pb. The toxicity trend of LC_{50} at 96 hpf observed was Hg (0.0217 mgL^{-1}) < Cu (0.099 mgL^{-1}) < Cd (0.407 mgL^{-1}) < Zn (14.021 mgL^{-1}) < As (34.840 mgL^{-1}) < Pb (41.697 mgL^{-1}). LC_{50} did not change over time in Cu, Zn and As (95% confidence limits) indicating the potential toxicity to early developmental stages in zebrafish embryos (Table 1).

Table 1. Summarized LC_{50} value of trace metals on *Danio rerio* embryo for a period of 24 – 96 hpf.

Trace metals	LC_{50} (95% confident limits – lower, upper)			
	24 hpf	48 hpf	72 hpf	96 hpf
Hg	0.0397 (0.0324,0.0488)	0.0229 (0.0245,0.0367)	0.0218 (0.0182,0.0263)	0.0217 (0.0180,0.0260)
Cu	0.1735 (0.1498,0.2011)	0.1306 (0.1128,0.1512)	0.0991 [a] (0.0844,0.1157)	0.0991 [a] (0.0844,0.1157)
Cd	0.5660 (0.4339,0.741)	0.464 (0.3505,0.613)	0.418 (0.3152,0.550)	0.407 (0.3072,0.536)
Zn	23.083 (18.871,28.13)	17.080 (13.936,20.801)	14.021 [b] (11.347,17.147)	14.021 [b] (11.347,17.147)
As	42.915 (36.223,50.97)	38.313 (32.007,45.98)	34.840 [c] (28.903,42.08)	34.840 [c] (28.903,42.08)
Pb	113.8 (88.5,149.6)	63.90 (50.81,81.0)	43.75 (35.75,53.71)	41.70 (33.97,51.34)

[a] There is no difference in the LC_{50} value of the Cu at 72 and 96 hpf after exposure
[b] There is no difference in the LC_{50} value of the Zn at 72 and 96 hpf after exposure
[c] There is no difference in the LC_{50} value of the As at 72 and 96 hpf after exposure

In this study, the most toxic trace metal to zebrafish embryo was Hg. The LC_{50} values of Hg at 24, 48, 72 and 96 hpf were less than 0.04 mgL^{-1}. It shows that even at lower concentrations it can cause a high mortality rate on zebrafish embryos. Previous studies reported that LC_{50} of zebrafish embryos exposed to mercuric chloride ($HgCl_2$), at 96 hpf was 0.07 mgL^{-1} (Vutukuru and Basani, 2013). There are some studies on the toxicity effect of Hg on some fish species embryo, For example, LC_{50} value of Rainbow trout, channel catfish, Goldfish and Largemouth bass has been reported as below 0.7 μgL^{-1}, 0.3μgL^{-1}, 0.7 μgL^{-1} and 5.3 μgL^{-1} respectively (Boening, 2000).

Cu and Cd are also highly toxic to aquatic organisms. However, Cu caused more toxicity to the fish compared to Cd. The results of many studies showed higher toxicity of Cu compared to Cd (Zhu *et al*., 2011; Witeska *et al*., 2014; Yang, 2014) including the findings in this study. There is an increasing concern about the As contamination in the water. Various researchers use different animal models to investigate the basic mechanism of As mediated developmental toxicity (Rodriguez *et al*., 2002; Ghandi and Kumar, 2013). This study showed a higher LC_{50} value for the As compared to Hg, Cd, Zn and Cu indicating that As was less toxic to zebrafish embryos (Spehar *et al*., 1980). Pb lacks biological functions and is toxic for aquatic organisms even at a small dose (Osman, 2007). According to the toxicity assay, the least toxic metal on zebrafish embryos was Pb. However, early exposure to Pb may cause severe defects (Weis and Weis, 1977) which is also observed in this study.

Sub- Lethal and Teratogenic Effects

In the normal development of zebrafish embryo, somite formation starts after the 10 hpf, and the tail development, tailbud detachment from the yolk sac can be observed after 24 hpf followed by all the other major organ development which can be observed within five days. Hence, lack of somite formation and tail detachment are considered features of the dead embryos (Kimmel *et al*., 1995; Lammer, 2009).

During the zebrafish embryotoxicity test, several sub-lethal and teratogenic endpoints were observed in addition to the four major lethal endpoints. In negative and internal controls, sub-lethal or teratogenic endpoint was absent and normal age-specific developmental features such as straightening of the body axis from its curvature around the yolk sac, blood vascular circulation, heartbeat, and epidermal pigmentation were observed.

Zebrafish embryos at 72 hpf displayed normal development of all major organs in negative and internal control groups and the lowest concentration of As (2 mgL^{-1}, 4 mgL^{-1}), Pb (2 mgL^{-1} , 4 mgL^{-1}), and Zn (0.025 mgL^{-1} , 0.05 mgL^{-1}). However, several deformities were observed in the other treatment groups including growth retardation, shrinkage of chorion, scoliosis, pericardial edema, yolk sac edema, lack of pigmentation, tail deformities, hemorrhages, missing formation of lens - Placodes and lack of otoliths (Figure 3).

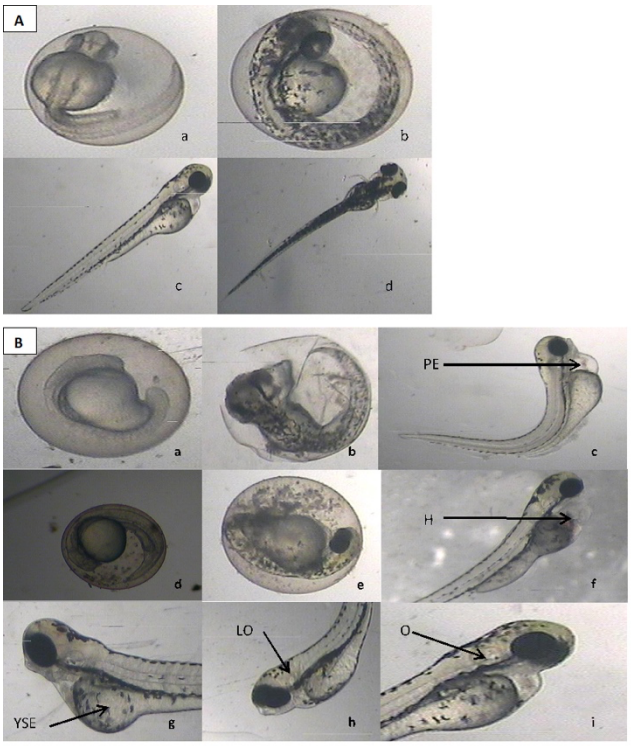

Figure 3. Normally developed zebrafish embryo after 24 hpf (a), 48 hpf (b), 72 hpf (c), and 96 hpf (d) (A). Sub-lethal and teratogenic endpoints ; (a) Growth retardation, (b) Shrinkage of chorion, (c) Scoliosis and Pericardial edema (PE), (d) Lack of pigmentation, (e) tail deformities, (f) Hemorrhages (H), (g) Yolk – sac edema (YSE), (h) Lack of otoliths (LO), (i) Normally developed zebrafish embryo (otolinth – O) after 96 hpf (B)..

Skeletal deformities in fish are a good bioindicator of pollution (Villeneuve *et al*., 2005). Damage of the vertebral column expressed as the curvature of the larval body axis (scoliosis) occurss due to almost all heavy metal toxicities (Osman, 2007). Scoliosis observed in this study on metal exposed embryos implicates the potential risk of toxicity to aquatic biota. Growth retardation was mainly present in embryos exposed to Cu and Cd, which is known to be the most powerful growth inhibitor causing damage to the vertebral column by inhibition of collagen synthesis (Sikorska and Wolnicki, 2006). Pb causes scoliosis by impairing ionic regulation (Słomińska and Jezierska, 2000; Khayatzadeh and Abbasi, 2010) while Cd, Pb, Zn and As are considered as neurotoxic, having the ability to fracture the vertebrae through tetanic muscular contraction (Villeneuve *et al*., 2005). Scoliosis was one of the main observations found in this study from all trace metals tested.

The tail deformities are caused by the inability of metal treated embryos to express the evenskippid gene, which is important during the tail development, while pigmentation of the zebrafish skin is controlled by Melanocyte Stimulating Hormone (αMSH) and Melanin-Concentrating Hormone (MCH). As a result of the metal treatment, synthesis of particular gene and hormones were impaired inhibiting the tail detachment and pigment development were inhibited (Osman, 2007). Hemorrhages usually occur as a result of fractures at the base of the neural or haemal arch of the centrum (Villeneuve *et al*., 2005). In this study, hemorrhages were observed mostly in the Pb treated embryos. Pericardial edema, which is a non-specific symptom seen in response to inorganic or organic

pollutants was also found in zebrafish embryos exposed to Zn, Cu, and As.

Lack of otolith formation was recorded in the embryos treated with Cd and Cu. Many metals directly enter the olfactory system where they can disrupt normal function by accumulating in and damaging cells of the olfactory system. It can disrupt the transmission of information from the olfactory lobes to higher levels of the brain (Weis, 2014).

Zebrafish embryos revealed a lot of toxic signs and symptoms indicating the risks to the aquatic environment by metal pollution even at a low concentration. Hence, the findings and the methods used in this study can be adhered to for water quality testing and determining aquatic environmental pollution.

CONCLUSION

Based on LC_{50} values, sub-lethal and teratogenic effects in zebrafish embryos, Hg was highly toxic followed by Cu, Cd, Zn, As and Pb. The LC_{50} value of each trace metal was increasing with the increasing concentration and Cu, Zn and As remained static after 72 hpf. Sub-lethal and teratogenic deformities such as growth retardation, lack of tail development, lack of eye lens (placode), yolk sac edema, pericardial edema, hemorrhages, shrinkage of chorion, and scoliosis were observed in metal treated embryos.

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