ZINC CONTAMINATION IS AN UNDERESTIMATED RISK TO AMPHIBIANS: TOXICITY EVALUATION IN TADPOLES OF FEJERVARYA LIMNOCHARIS

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Highlights

- ZnCl₂ produced concentration dependent mortality to F. limnocharis tadpoles.
- Sub-lethal ZnCl₂ altered metamorphosis time of tadpoles.
- ZnCl₂ induced DNA strand breaks and micronucleus in tadpoles.

Abstract. Aquatic environments are often contaminated with zinc. Amphibian tadpoles are likely to be exposed to high concentrations of zinc present in these environments. We determined the acute and sub-chronic toxicity of ZnCl₂ on Fejervarya limnocharis tadpoles under laboratory conditions. The LC₅₀ values of ZnCl₂ were found to be 5.81, 4.32, 3.79 and 3.61 mg/L at 24, 48, 72 and 96 h of exposure respectively. Long-term exposure to sub-lethal concentrations of ZnCl₂ induced significant mortality in concentration and time dependent manner. Sub-lethal ZnCl₂ exposure significantly altered survival, body length and body weight at metamorphosis. Micronucleus test and comet assay indicated the genotoxic potential of ZnCl₂. Significant increase in DNA strand break was observed following ZnCl₂ exposure equivalent to 1% of the of 24 h LC₅₀ value. The findings indicate possible adverse to tadpoles inhabiting aquatic environments contaminated with zinc. In addition, the findings may be extrapolated to aquatic organisms of similar trophic status.

Keywords: zinc, Fejervarya limnocharis, genotoxicity, micronucleus, comet assay.

Introduction

Heavy metal contamination of aquatic environment is one of the common and persistent forms of pollution. Heavy metals have been identified as one of the significant causative factors of ecological degradation in aquatic habitats (Baldantoni et al., 2004). Aquatic environments are polluted by heavy metals due to natural processes through weathering and leaching of mineral deposits (Purushothaman & Chakrapani, 2007; Adamu et al., 2015; Skordas et al., 2015) as well as human economic activities (Mohiuddin et al., 2011; Wei & Yang, 2010).

Zinc is an essential element required for normal metabolic process (Vladimirov, 1969; Frieden, 1972). Besides, zinc is widely used in industry for manufacture of a broad range of products ranging from paints to pharmaceuticals and cosmetics. Other common uses of zinc for economic activities include metal plating, plastic production, electrical components and battery manufacturing. The ambient natural background concentration of zinc in freshwater bodies is less than 50 μg/liter. However, concentrations up to 4 mg/liter in water and 100 mg/kg dry weight in sediments have been reported in anthropogenically contaminated freshwater habitats (World Health Organization [WHO], 2001; Mondal et al., 2017; Sarkar et al., 2017). Heavy metals are toxic to living organisms. But, unlike other heavy metals such as copper, cadmium, mercury, lead and the metalloid arsenic, zinc has always been considered an underestimated risk factor for aquatic organisms. There are studies, though limited in number, suggesting that aquatic organisms exposed to higher concentrations of zinc could exhibit significant adverse
physiological effects (Sinley et al., 1974; Benoit & Holcombe, 1978; Holcombe et al., 1979; Leland, 1983; WHO, 2001; Brinkman & Woodling, 2005; Bringolf et al., 2006).

Amphibians are an important group of vertebrates occupying critical positions in many food chains. In fact, in wetland ecosystems, these are often regarded as key stone species. A global assessment has revealed that amphibians are declining rapidly and up to 40% of the species have been affected in this process (Stuart et al., 2004). Environmental pollution has been identified as one of the major factors of such decline in amphibian species and population. The larval stages of amphibians are spent in aquatic habitats especially in shallow ephemeral ponds. Due to shorter water columns in these habitats, the tadpoles of amphibians spent a significant period in the bottom sediments to avoid daytime increase in water temperature. Therefore, they are vulnerable to contaminants present in the water column as well as the pollutant rich bottom sediments.

The worldwide decline in amphibian population has attracted increasing attention from scientists in recent years (Beebee & Griffiths, 2005). Several studies have shown that heavy metals adversely produce lethal and sub-lethal toxicity in amphibians. Surprisingly, little or no information is available on the possible toxic effects of zinc in anuran amphibians. In the present study, we have examined the effects of zinc on the tadpoles of *F. limnocharis*. The systematic analysis of multiple toxicological endpoints covering acute toxicity, changes in life history traits and genotoxicity provides important toxicological insights into this otherwise lesser-known heavy metal in amphibians.

1. **Materials and methods**

1.1. **Collection, rearing and maintenance of study animal**

*F. limnocharis* tadpoles were collected from an artificial captive breeding pond near the Assam University, Silchar campus which is not contaminated by any source of contaminant exposure. Tadpole rearing was done as described earlier (Giri et al., 2012). Prior to experiments, the tadpoles were subjected to acclimation in the laboratory in aerated medium for 48-h. These were screened to identify and separate the tadpoles belonging to different Gosner stages (Gosner, 1960). The experiments were performed at 26±1 °C and 12-h light and dark cycles. Grinded fish food were used to feed the tadpoles without any restriction. Chemically pure salts of ZnCl₂ dissolved in distilled water was used as the test agent. ZnCl₂ (mol wt. 136.30; ≥95% pure, CAS Registry No. 7646-85-7) were purchased from Merck Specialities Private Limited, Mumbai, India. The study has ethical clearance of the Assam University through approval letter AUS/IAEC/2017/PC/02.

1.2. **Acute toxicity studies and determination of LC₅₀**

Acute toxicity experiments were performed in polypropylene tubs containing 2 L of aged well water. Each tub housed 10 tadpoles. The tadpoles belonging to Gosner stage 22–25 were subjected to either no treatment or exposed to four different concentrations (3, 4, 5 and 6 mg/L) of ZnCl₂. The five treatment conditions were replicated thrice for a total of 15 experimental units. At 24 h intervals, for the next 96 h, experimental tubs were monitored and any dead individuals were carefully removed keeping record for each. The tadpole survival data was used to calculate the LC₅₀ values at different time points using probit analysis.

1.3. **Chronic exposure and toxicity studies**

Chronic toxicity evaluations were also made in polypropylene tubs following sub-lethal ZnCl₂ concentrations over longer period of time. The tadpoles of Gosner developmental stage 22–25 were exposed to four different sub-lethal concentrations (0.5, 1.0, 1.5 and 2.0 mg/L) of ZnCl₂ approximately ranging between 10% and 35% of the 24 h LC₅₀ values. The control groups were not exposed to any kind of treatment. The five treatment conditions were replicated thrice for a total of 15 experimental units. The tub water was changed every alternate day and ZnCl₂ was reapplied in to the respective tubs. The experiments were terminated following either death or metamorphosis of all individuals in the experimental groups. Survival status of the tadpoles recorded on daily basis and deceased ones were removed. On day 23, the first metamorphosis occurred. Therefore, the tadpole survival data for the first 23 days of the exposure period among various treatment groups were compared. In addition, survival percentage at metamorphosis as well as average time to metamorphosis in each group was determined. In addition, the average body weight as well as snout to vent length (SVL) of the newly metamorphosed froglets were measure in each treatment group. The metamorphosed froglets were examined for major morphological defects if any and noted. The water parameters were regularly monitored during the course of the experiments. Dissolved oxygen content was always >8.4 mg/L and pH varied between 7.4 and 7.6.

Kaplan–Meier test was used to compare the survival percentage among the treatment groups. Time to metamorphosis as well as morphometric parameters such as SVL and body weight of the metamorphosed individuals were analyzed using ANOVA. Post hoc analysis (Tukey’s–HSD) was also performed to compare among the treatment groups. Statistical analyses were performed at 95% confidence interval using the 18.0 version of SPSS statistical software.

1.4. **Micronucleus test**

Amphibian erythrocytes are nucleated and multiply in the circulation during larval stages (Duellman & Trueb, 1986). Therefore, erythrocytes cells are suitable for micronuclei (MN) detection which can be readily counted in blood smears (Campana et al., 2003; Giri et al., 2012). The MN assay was performed in peripheral blood erythrocytes as described previously (Giri et al., 2012). The tadpoles of...
Gosner stage 26–28 were selected. During this developmental period, intense hematopoiesis takes place which is suitable for genotoxicity studies. This experiment was performed in polypropylene tubs containing 2 L of aged well water as described earlier. The tadpoles were exposed to four different concentrations of ZnCl₂ (0.5, 1.0, 1.5 and 2.0 mg/L). Negative (no treatment) and positive (cyclophosphamide 2 mg/L) control groups were included with the exposure groups. The six treatment conditions were replicated thrice for a total of 18 experimental units. After 24, 48, 72 and 96-h of the treatments, 5 live tadpoles from each group were anesthetized in 4% buffered MS222. At least 2 smears per tadpole were made with peripheral blood. The blood smears were fixed in absolute methanol for 3 min and air-dried. A day later the slides were coded and stained in buffered Giemsa (10%). Analysis of MN was carried out in 1000 cells per tadpole under the microscope at a final magnification of 1000X. The scoring criteria was similar to those described by Lajmanovich et al. (2005). ANOVA was used to analyze change in MN frequency at different concentration levels and time points. Treatment effects on MN frequency was assessed using linear regression analysis.

1.5. Comet assay

This experiment was performed for the investigation of DNA damage (single-, double-strand breakage) under alkaline condition at the individual cell level by following the protocol of Singh et al. (1988) with subsequent modifications of Tice et al. (2000). In brief, tadpoles Gosner stage 26–28 were exposed to 58.08 µg/L (1% of 24-h LC₅₀ value) of ZnCl₂. The use of this concentration is intended to determine the genotoxic potential of ZnCl₂ at environmentally relevant concentration which otherwise may not be detected by the MN test. There were 6 tadpoles in each treatment group (3 in each experiment repeated twice). Cardiac blood collected following 24-h of exposure was mixed with calcium and magnesium free PBS (pH 7.4). An aliquot of cell suspension containing 10⁶ cells/ml was diluted in low melting agarose in a ratio of 1:10. Aliquots of 85 µl of the mixture were rapidly spread on precoated frosted slides and allowed to polymerize in dark. Then, the slides were immersed in freshly prepared ice-cold lysing solution (pH 10) containing 10 mM Trizma base, 10% DMSO, 100 mM Na₂EDTA, 2.5 M NaCl, 1% TritonX100. DNA unwinding process was allowed to take place for 20 minutes at pH 13.5 in fresh electrophoresis buffer consisting of 300 mM NaOH in 1 mM Na₂EDTA in the electrophoresis chamber. Electrophoresis was carried out at a constant voltage of 24 V and 300 mA at 4 °C for 20 min. Then the slides were transferred to the neutralizing buffer (Tris-HCl, pH 7.5) and kept in dark. The neutralizing solution was changed at 5 minutes intervals for thrice. The slides were stained in 20 µg/ml EtBr followed by rinsing in double distilled water to remove the unbound EtBr. Kinetic imaging image analysis system (Komet 5.5, Andor Technology, Nottingham, UK) was used for quantitative analysis of DNA damage in the cells. A charge coupled device (CCD) camera as part of Leica fluorescence microscope (Wetzlar, Germany) was used to acquire the images for analysis by the software. The final magnification was 400x. Comet data was analyzed using 2-tailed Student’s t-test.

2. Results

2.1. Acute toxicity studies and determination of LC₅₀

The acute LC₅₀ values of ZnCl₂ in F. limnocharis were found to be 5.81, 4.32, 3.79 and 3.61 mg/L respectively at 24, 48, 72 and 96 h (Table 1). None of the animals in the control group died. As the exposure period increased, the LC₅₀ values decreased in a linear manner. Linear regression analysis of the mean lethal concentration showed significant (R² = 0.851, p < 0.05) concentration and time effect.

| Duration of exposure | LC₅₀ value (mg/L) |
|----------------------|-------------------|
| 24 hour              | 5.81              |
| 48 hour              | 4.32              |
| 72 hour              | 3.79              |
| 96 hour              | 3.61              |

2.2. Chronic toxicity studies on tadpole survival, growth and development

Tadpoles of F. limnocharis exposed to sublethal concentrations of ZnCl₂ caused increased rate of mortality which was both concentration and time dependent (Figure 1). In the overall comparison of the 23-days tadpole survival data, ZnCl₂ had significant (p < 0.001) effect as revealed by Kaplan–Meier product limit estimate. ZnCl₂ at highest concentration (2 mg/L) used in the present study could exhibit only 33% survival up to day 23 following the exposure.

In comparison to the control, the metamorphosis time in the groups exposed to sub-lethal concentrations of ZnCl₂ increased by 10% (Table 1). The final magnification was 400x. A charge coupled device (CCD) camera as part of Leica fluorescence microscope (Wetzlar, Germany) was used to acquire the images for analysis by the software. The final magnification was 400x. Comet data was analyzed using 2-tailed Student’s t-test.

Figure 1. Survival of tadpoles of F. limnocharis after 23d of exposure to different sub-lethal concentrations of ZnCl₂. Values are mean ± SE
of ZnCl₂ was significantly delayed (Figure 2). The metamorphosis pattern was monitored up to 50 days till all of the tadpoles either metamorphosed or died due to toxicity. Tadpoles exposed to the lowest concentration of 0.05 mg/L of ZnCl₂ took significantly more time to metamorphose. However, those exposed to 2 mg/L was failed to metamorphose and caused 100% mortality within 28 days of the exposure (Figure 2). The average metamorphosis time in the exposed group receiving 0.5 mg/L of ZnCl₂ was significantly higher (p < 0.05) as compared to control group. Tadpoles in the control group took an average time of 28.87±1.42 days for metamorphosis.

Tadpoles exposed to highest concentrations of ZnCl₂ such as 2 mg/L did not survive till metamorphosis (Figure 3). The number of tadpoles which survived till metamorphosis was dependent on the concentration of ZnCl₂ (one-way ANOVA, F_{4, 70}= 390.026, p < 0.001).

The average body weight of the metamorphosed froglets in the ZnCl₂ exposed groups was found to be apparently higher than in the control group (Figure 4A). However, these were not statistically significant. The snout to vent length of the metamorphosed froglets is often used as standard measure of body length indicative of skeletal growth. In contrast to body weight, Tukey’s pair wise comparison test indicated that at lower concentrations, zinc chloride caused significant (p < 0.05) increased in snout vent length of metamorphosed froglets at metamorphosis (Figure 4B). ZnCl₂ in the concentration ranges tested did not cause any other apparent malformations in any of the exposed groups. However, a few cases of abdominal edema were observed in the groups exposed higher concentrations of ZnCl₂.

Table 2. Incidence of micronucleated erythrocytes induced by ZnCl₂ in tadpoles\(^{a,b,c}\)

| Concentration | Exposure period |
|---------------|----------------|
|               | 24 h | 48 h | 72 h | 96 h |
| Control       | 0.30±0.06 | 0.25±0.13 | 0.33±0.06 | 0.25±0.13 |
| CP 2 mg/L     | 11.67±0.66 | 13.93±0.42 | 13.47±0.49 | 13.13±0.53 |
| Zinc chloride |       |       |       |       |
| 0.5 mg/L      | 0.33±0.10 | 0.45±0.10 | 0.56±0.06* | 0.45±0.10 |
| 1.0 mg/L      | 0.40±0.06 | 0.80±0.13* | 0.93±0.13** | 1.00±0.16*** |
| 1.5 mg/L      | 0.60±0.06* | 1.18±0.19*** | 1.20±0.13*** | 1.33±0.06*** |
| 2.0 mg/L      | 1.00±0.13*** | 1.45±0.06*** | 1.50±0.13*** | 1.53±0.10*** |

Note: \(^{a}\) Control: no treatment was given; \(^{b}\) CP: cyclophosphamide (positive control); \(^{c}\) Values are frequency of micronucleated erythrocytes (%) expressed as means ± SE based on 1000 cells per animal (n = 15); \(^{*}\) Level of significance from respective control values: \(^{*}\) = p < 0.05; \(^{**}\) = p < 0.01; \(^{***}\) = p < 0.001. Statistical analysis: ANOVA.

Figure 2. Time taken by tadpoles to metamorphose following exposure to different concentrations of ZnCl₂. Data are significantly different from control (ANOVA). (*) = p < 0.05

Figure 4. ZnCl₂ induced changes body weight (A) and snout to vent length (B) of froglets at metamorphosis. Data are significantly different from the control group at p < 0.05 (*)
2.3. Micronucleus test

ZnCl₂ exposure induced MN in the erythrocytes of *F. limnocharis* tadpoles (Table 2) at 24 h (F₄, 70 = 9.82, p < 0.05), 48 h (F₄, 70 = 33.78, p < 0.01), 72 h (F₄, 70 = 42.46, p < 0.001), and 96 h (F₄, 70 = 47.53, p < 0.01). There were significant positive correlations between the concentrations of ZnCl₂ and micronucleus frequency (Figure 5). The correlation coefficients at 24 h, 48 h, 72 h and 96 h were 0.9117 (p < 0.01), 0.9955 (p < 0.001), 0.9981 (p < 0.001) and 0.9864 (p < 0.001) respectively. In the time response study, except for 0.5 mg/L of ZnCl₂ (r = 0.6361) all the other treatments tested showed time dependent increase in the frequency of MN all through the 96th study period. Moreover, it was found that that the overall time effect on micronucleus induction (ANOVA) was statistically significant (F₄, 295 = 4.82, p < 0.05).

2.4. Comet analysis

Erythrocytes of *F. limnocharis* tadpoles showed significant change in the degree of DNA damage following ZnCl₂ exposure as evidenced by changes in comet parameters (Figure 6). Quantitative analysis revealed that amount of DNA...
present in comet head region significantly decreased (p < 0.05) with concurrent increase in the tail region (p < 0.05) compared to the control group. Olive tail moment (OTM) in a comet represents the product of the amount of DNA in the tail region as well as the average distance they migrate in the gel. In the present study, the OTM in the ZnCl₂ exposed cells was significantly higher (p < 0.05) compared to the control cells.

3. Discussion

The present study evaluated acute and subchronic toxicity of ZnCl₂ in tadpoles of *F. limnocharis*. It was found that tadpole mortality rate was positively correlated with exposure to ZnCl₂ concentration. The calculated 96 h LC₅₀ value of ZnCl₂ in the current study was found to be 3.61 mg/L (Table 1). Similar LC₅₀ value was also found in the earlier studies by Svecevičius (1999). Bagdonas and Vosylienė (2006) reported that the 96 h LC₅₀ value of zinc in Rainbow trout (*Onchorhyncus mykiss*) was 3.79 mg/L. The LC₅₀ values for ZnCl₂ found in the present study are similar to those previously reported in tadpoles by Khangarot and Ray (1987) and Shuhaimi-Othman et al. (2012). The LC₅₀ values for zinc have been shown to vary over a wide range depending upon the species and developmental stages. For example, the 96 h LC₅₀ values was reported to be 2.1 mg/L for *Rana hexadactyla* and 28.38 mg/L for *Rana luziventris* (Khangarot et al., 1985; Lefcort et al., 1998). However, available literature also reported that the most published LC₅₀ values for other amphibian tadpoles are greater than 19 mg/L (Linder & Grillitsch, 2000). One possible reason for this wide range of LC₅₀ data is due to the fact that toxicity of Zn ions is highly dependent on water hardness; the highest LC₅₀ value available in the literature was reported in the current study was found to be 3.61 mg/L.

In the present study, long term exposure at sublethal concentrations (0.5–2.0 mg/L) of ZnCl₂ to *F. limnocharis* tadpoles demonstrate that the percentage of tadpole survival decreased significantly with increasing metal concentrations (Figure 1). Interestingly, there was a significant interaction between increasing ZnCl₂ concentration and the duration of exposure of the tadpoles. Tadpoles exposed to highest sub-lethal concentration of ZnCl₂ (2 mg/L) did not survive till metamorphosis which suggests that tadpole survival was dependent on metal treatment (Figure 2). This may be due to the fact that the reduced growth rates of tadpoles at high metal concentrations are caused by increased metabolic costs, which leaves little energy for growth (Rowe et al., 1998). Studies extending for longer periods have shown that metal exposure reduces tadpole survival to metamorphosis (Lefcort et al., 1998). Our results demonstrate that sub-lethal concentrations of ZnCl₂ significantly delayed the time to metamorphosis process (Figure 3). Similar findings have been reported in earlier studies with zinc and copper metal ion exposure on the germination of frogs spawn and on growth of tadpole (Dilling & Healey, 1926). Lefcort et al. (1998) reported that low levels of lead, zinc and cadmium did not significantly delay time to metamorphosis, but the low lead and low zinc exposed animals underwent metamorphosis at a lower mass than control tadpoles. In fishes such as fathead minnow (Brungs, 1969), zebrafish (Dave et al., 1987) and the flounder *Paralichthys olivaceus* (Yulin et al., 1990), zinc has been reported to delay the time-to-hatch.

The standard measurement of body length is an important parameter to determine the skeletal growth of metamorphosed froglets at metamorphosis. Our study demonstrate that at low concentrations (0.5 and 1.0 mg/L) of ZnCl₂ significantly increased body length of metamorphosed froglets (Figure 4B). Contrary to our findings, some reports suggest that body length of metamorphosed froglets is not influenced by exposure to lower concentrations of zinc. However, as metal concentrations increased, tadpole body length decreased significantly (Lefcort et al., 1998; Haywood et al., 2004). Therefore, it is evident that there exists species specific sensitivity among different anuran species to a given toxicant. Morphological and physiological abnormalities in amphibians exposed to toxicants have been well-studied (Stebler et al., 1988; Bantle et al., 1989; Hopkins et al., 2000). However, the mechanisms by which zinc influences amphibian metamorphosis remain unclear.

The micronucleus test in erythrocytes of anuran tadpoles is widely used in experimental models for the biomonitoring studies as a sensitive biomarker of environmental contaminant induced genotoxicity in aquatic organisms. MN test has served as an index of cytogenetic damage for over 30 years (Fenech et al., 2003). In the present study, ZnCl₂ was found to be genotoxic in the micronucleus test in tadpoles of *F. limnocharis*. It was observed that the frequency of micronucleus increased with increasing exposure concentration of ZnCl₂ (Table 2). Similar findings have been reported in earlier studies (Wei et al., 2015) in *Rana zhenhaiensis* tadpoles exposed to Zn⁺². Earlier studies by Bagdonas and Vosylienė (2006) reported genotoxicity of Cu, Zn in MN test in rainbow trout erythrocytes; but there were no dose-dependent changes in micronucleated erythrocytes. Similar result has been found in our previous studies with cadmium chloride exposure on *Rana limnocharis* tadpoles (Patar et al., 2016). The present findings are in agreement with majority of previously reported studies with pesticides and heavy metals in *X. laevis larvae*, *R. limnocharis*, *E. cyanophyllys* and *Bullfrog* tadpoles (Mouchet et al., 2006; Giri et al., 2012; Yadav et al., 2013; Montalvão & Malafaia, 2017). Apart from amphibian tadpoles, MN test in experimental fish models have been well-documented. Obiakor et al. (2010) conducted MN test on *Synodontis clarias* and *Tilapia nilotica* species and reported that zinc exposure caused significant increase in the frequency of micronucleated erythrocytes.
produced in both the species. Bakar et al. (2014) demonstrated that zinc exposure to *Oreochromis niloticus* species produced significant increased induction of MN and erythrocytes with nuclear abnormalities compared with the control group.

MN induction is an indicator of altered cytogenetic effects reflecting changes in chromosome number and/or structure. These lost chromosome(s) or chromosomal fragment(s) fail to participate in the anaphasic movement, thus fail to be part of the main nucleus (Muranli & Güner, 2011). On the other hand, comet assay (CA) is used to detect double or single DNA strand breaks in the interphase nuclei. CA is widely used in field monitoring and in laboratory experiments to demonstrate the sensitivity of aquatic organisms to genotoxic agents (Clements et al., 1997; Mouchet, 2002; Mouchet et al., 2005, 2007; Frenzilli et al., 2009; Singha et al., 2014; Patar et al., 2016). In the present study, ZnCl₂ exposed groups clearly demonstrate that zinc induces a considerable amount of DNA strands breaks in *F. limnocharis* at very low concentration. The DNA damage is indicated by significant alterations in various comet parameters (Figure 6). Compared to other heavy metals namely Cd and Cu; studies on the genotoxic potential of zinc in amphibian tadpoles using comet assays are infrequent. However, using this sensitive tool, genotoxic potential of zinc have been shown in various model organisms such as fish and mice as well as in human cells (Banu et al., 2001; Ho & Ames, 2002; Ho et al., 2003; Zhang et al., 2008; Sliwinski et al., 2009). All in all, the present findings on genotoxicity analysis suggest the genotoxic potential of ZnCl₂ in *F. limnocharis* tadpoles.

Several studies have assessed the genotoxicity of zinc chloride following oral or parental exposure in various multicellular organisms. *In vitro* studies have shown that zinc exposure to induce DNA damage. Using comet assay Banu et al. (2001) have shown that zinc produces DNA single strand breaks in *in vivo*. In human lung cells, it has been shown that DNA double strand breaks as well as chromosomal instability occur following exposure to higher concentrations of zinc (Xie et al., 2009). Similar effects have been shown in bone marrow cells following zinc exposure in *in vivo* (Vilkina et al., 1978). Kowalska-Wochna et al. (1988) reported that zinc chloride given to rats in drinking water at a dose rate of 14.8 mg/kg/day caused significant damage to the gentic material. Genotoxic effects of zinc administered either intraperitoneally (Gupta et al., 1991) or by inhalation (Voroshilin et al., 1978) have also been reported in mice test system. However, the Agency for Toxic Substances and Disease Registry [ATSDR] (1990) report provides indication of zinc to be a weak clastogenic agent. Several studies also have reported that high zinc concentrations can interfere with ROS detoxification processes and thus contributes to ROS accumulation (Nzengue et al., 2011). However, the underlying molecular mechanism of zinc-induced genotoxicity is poorly understood and requires further investigations.

**Conclusions**

In conclusion, this present study provides important information regarding acute and sub-chronic toxicity of ZnCl₂ to larval amphibians adding to the present scientific knowledge. But; there is a paucity of information about sub-lethal effects of zinc on the early stages of amphibian development. Therefore, further investigations are essential using more different sub-lethal concentrations of ZnCl₂ in aquatic organisms especially in amphibian. All in all, this study suggests the possible role of heavy metal pollution such as zinc towards amphibian population decline and could have similar effects in other aquatic organisms.

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**Author contributions**

The contribution of authors are as follows. AP – planning, experiments, analysis and manuscript writing; ID – experiments and analysis; SG – planning, evaluation and manuscript writing; AG – planning, data analysis and manuscript writing.

**Conflict of interest statement**

The authors declare no conflict of interest.

**References**

Adamu, C., Nganje, T., & Edet, A. (2015). Heavy metal contamination and health risk assessment associated with abandoned barite mines in Cross River State, southeastern Nigeria. *Environmental Nanotechnology, Monitoring & Management*, 3, 10–21. https://doi.org/10.1016/j.enmm.2014.11.001

Agency for Toxic Substances and Disease Registry. (1990). *Toxicological profile for zinc*. ATSDR, Atlanta, Georgia, USA. https://www.atsdr.cdc.gov/toxprofiles/tp60.pdf

Bagdonas, E., & Vosyliene, M. Z. (2006). A study of toxicity and health risk assessment associated with abandoned barite mines in Cross River State, southeastern Nigeria. *Environmental Nanotechnology, Monitoring & Management*, 3, 10–21. https://doi.org/10.1016/j.enmm.2014.11.001

Bakar, S., Ashriya, A., Shuib, A., & Razak, S. (2014). Genotoxic effect of zinc and cadmium following single and binary mixture exposures in tilapia (*Oreochromis niloticus*) using micro-nucleus test. *Sains Malaysia*, 43(7), 1053–1059.

Baldantoni, D., Alfani, A., Di Tommasi, P., Bartoli, G., & De Santo, A. V. (2004). Assessment of macro and microelement accumulation capability of two aquatic plants. *Environmental Pollution*, 130(2), 149–156. https://doi.org/10.1016/j.envpol.2003.12.015

Bantle, J. A., Fort, D. J., & James, B. L. (1989). Identification of developmental toxicants using the Frog Embryo Tera-
A. Patar et al. Zinc contamination is an underestimated risk to amphibians: toxicity evaluation in tadpoles of...

Frieden, E. (1972). The chemical elements of life. *Scientific American*, 227, 52–64.

Giri, A., Yadav, S. S., Giri, S., & Sharma, G. D. (2012). Effect of predator stress and malathion on tadpoles of Indian skittering frog. *Aquatic Toxicology*, 106–107, 157–163. https://doi.org/10.1016/j.aquatox.2011.11.008

Gosner, K. L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica*, 16(3), 183–190.

Gupta, T., Talukder, G., & Sharma, A. (1991). Cytotoxicity of zinc chloride in mice in vivo. *Biological Trace Element Research*, 30, 95–101. https://doi.org/10.1007/BF02990346

Haywood, L. K., Alexander, G. J., Byrne, M. J., & Cukrowska, E. (2004). *Xenopus laevis* embryos and tadpoles as models for testing for pollution by zinc, copper, lead and cadmium. *African Zoology*, 39(2), 163–174. https://doi.org/10.1080/15627020.2004.11657213

Ho, E., & Ames, B. N. (2002). Low intracellular zinc induces oxidative DNA damage, disrupts p53, NFKB, and API DNA binding, and affects DNA repair in a rat glioma cell line. *Proceedings of the National Academy of Sciences*, 99(26), 16770–16775. https://doi.org/10.1073/pnas.222679399

Ho, E., Courtemanche, C., & Ames, B. N. (2003). Zinc deficiency induces oxidative DNA damage and increases p53 expression in human lung fibroblasts. *The Journal of Nutrition*, 133(8), 2543–2548. https://doi.org/10.1093/jn/133.8.2543

Holcombe, G. W., Benoit, D. A., & Leonard, E. N. (1979). Long term effects of zinc exposures on brook trout (*Salvelinus fontinalis*). *Transactions of the American Fisheries Society*, 108(1), 76–87. https://doi.org/10.1577/1548-8659(1979)108<76:LEZEOE>2.0.CO;2

Hopkins, W. A., Congdon, J., & Ray, J. K. (2000). Incidence and impact of axial malformations in larval bullfrogs (*Rana catesbeiana*) developing in sites polluted by a coal-burning power plant. *Environmental Toxicology and Chemistry*, 19(4), 862–868. https://doi.org/10.1002/etc.5620190412

Khangarot, B., & Ray, P. (1987). Sensitivity of toad tadpoles, *Buto melanosictus* (Schneider), to heavy metals. *Bulletin of Environmental Contamination and Toxicology*, 38, 523–527. https://doi.org/10.1007/BF01606623

Khangarot, B., Sehgal, A., & Bhasin, M. (1985). "Man and Biosphere" – Studies on the Sikkim Himalayas. Part 5: Acute toxicity of selected heavy metals on the tadpoles of *Rana hedactyla*. *Acta hydrochimica et hydrobiologica*, 13(2), 259–263. https://doi.org/10.1007/BF01950322

Kowalska-Wochna, E., Moniuszko-Jakoniuk, J., Kulikowska, E., & Miniuk, K. (1988). The effect of orally applied aqueous solutions of lead and zinc on chromosome aberrations and induction of sister chromatid exchanges in the rat (*Rattus sp.*). *Genetica Polonica*, 29(2), 181–189.

Lajmanovich, R. C., Cabagna, M., Peltzer, P. M., Stringhini, G. A., & Attademo, A. M. (2005). Micronuclear induction in erythrocytes of the *Hyla pulchella* tadpoles (Amphibia: *Hylidae*) exposed to insecticide endosulfan. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 587(1–2), 67–72. https://doi.org/10.1016/j.mrgentox.2005.08.001

Lefcort, H., Meguire, R., Wilson, L., & Ettinger, W. (1998). Heavy metals alter the survival, growth, metamorphosis, and predatory behavior of Columbia spotted frog (*Rana luteiventris*) tadpoles. *Archives of Environmental Contamination and Toxicology*, 35, 447–456. https://doi.org/10.1007/s002449900401
Leland, H. V. (1983). Ultrastructural changes in the hepatocytes of juvenile rainbow trout and mature brown trout exposed to copper or zinc. *Environmental Toxicology and Chemistry, 2*(3), 353–368. https://doi.org/10.1002/etc.5620020312

Linder, G., & Grillitsch, B. (2000). Ecotoxicology of metals. In D. W. Sparling, G. Linder, & C. A. Bishop (Eds.), *Ecotoxicology of amphibians and reptiles* (pp. 325–459). SETAC Press.

Mouhiddin, K., Ogawa, Y., Zakir, H., Otomo, K., & Shikazono, N. (2011). Heavy metals contamination in water and sediments of an urban river in a developing country. *International Journal of Environmental Science & Technology, 8*, 723–736. https://doi.org/10.1007/BF03326257

Mondal, P., Reichelt-Brushett, A. J., Jonathan, M. P., Sujitha, S. B., & Sarkar, S. K. (2017). Pollution evaluation of total and acid-leachable trace elements in surface sediments of Hooghly River Estuary and Sundarban Mangrove Wetland (India). *Environmental Science and Pollution Research, 25*(6), 5681–5699. https://doi.org/10.1007/s11356-017-0915-0

Montalvão, M. F., & Malafaia, G. (2017). Effects of abamectin on bullfrog tadpoles: insights on cytoxicity. *Environmental Science and Pollution Research, 24*, 23411–23416. https://doi.org/10.1007/s11356-017-0124-x

Mouchet, F. (2002). Validation du test comete sur larves d'amphibiens (xenopus laevis et Pleurodeles Walti) et application a l'évaluation du potentiel génotoxic de sols, sédiments et déchets contaminés. Comparaison avec filetest micronoyau amphibien [These de doctorat de l'Université Paul Sabatier de Toulouse]. Centre de Biologie du Développement.

Mouchet, F., Baudrimont, M., Gonzalez, P., Cuenson, Y., Bourdaine, J.-P., Boudou, A., & Gauthier, L. (2006). Genotoxic and stress inductive potential of cadmium in *Xenopus laevis* larvae. *Aquatic Toxicology, 78*(2), 157–166. https://doi.org/10.1016/j.aquatox.2006.02.029

Mouchet, F., Gauthier, L., Baudrimont, M., Gonzalez, P., Mailhes, C., Ferrier, V., & Devaux, A. (2007). Comparative evaluation of the toxicity and genotoxicity of cadmium in amphibian larvae (*Xenopus laevis* and *Pleurodeles waltl*) using the comet assay and the micronucleus test. *Environmental Toxicology, 22*(4), 422–435. https://doi.org/10.1002/tox.20267

Mouchet, F., Gauthier, L., Mailhes, C., Ferrier, V., & Devaux, A. (2005). Comparative study of the comet assay and the micronucleus test in amphibian larvae (*Xenopus laevis*) using benzo (a) pyrene, ethyl methanesulfonate, and methyl methanesulfonate: Establishment of a positive control in the amphibian comet assay. *Environmental Toxicology, 20*(1), 74–84. https://doi.org/10.1002/tox.20080

Murani, F. D. G., & Güner, U. (2011). Induction of micronuclei and nuclear abnormalities in erythrocytes of mosquito fish (*Gambusia affinis*) following exposure to the pyrethroid insecticide lambda-cyhalothrin. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 726*(2), 104–108. https://doi.org/10.1016/j.mrgentox.2011.05.004

Nzengué, Y., Candéias, S. M., Sauvaigo, S., Douki, T., Favier, A., Rachidi, W., & Guiraud, P. (2011). The toxicity redox mechanisms of cadmium alone or together with copper and zinc homeostasis alteration: its redox biomarkers. *Journal of Trace Elements in Medicine and Biology, 25*(3), 171–180. https://doi.org/10.1016/j.jtemb.2011.06.002

Obiakor, M., Okonkwo, J., Ezeonyejiaku, C., & Ezenwelu, C. (2010). Genotoxicology: Single and joint action of copper and zinc to *Synodontis clarias* and *Tilapia nilotica*. *Journal of Applied Sciences and Environmental Management, 14*(3), 59–64. https://doi.org/10.4314/jasem.v14i3.61468

Patar, A., Giri, A., Boro, F., Bhuyan, K., Singh, U., & Giri, S. (2016). Cadmium pollution and amphibians—Studies in tadpoles of *Rana limnocharis*. *Chemosphere, 144*, 1043–1049. https://doi.org/10.1016/j.chemosphere.2015.09.088

Purushothaman, F., & Chakrapani, G. (2007). Heavy metals fractionation in Ganga River sediments, India. *Environmental Monitoring and Assessment, 132*, 475–489. https://doi.org/10.1007/s10661-006-9550-9

Rowe, C. L., Kinney, O. M., Nagle, R. D., & Congdon, J. D. (1998). Elevated maintenance costs in an anuran (*Rana catesbeiana*) exposed to a mixture of trace elements during the embryonic and early larval periods. *Physiological and Biochemical Zoology, 71*(1), 27–35. https://doi.org/10.1086/515885

Sarkar, S. K., Mondal, P., Biswas, J. K., Kwon, E. E., Ok, Y. S., & Rinklebe, J. (2017). Trace elements in surface sediments of the Hooghly (Ganges) estuary: Distribution and contamination risk assessment. *Environmental Geochemistry and Health, 39*(6), 1245–1258. https://doi.org/10.1007/s10653-017-9952-3

Shulaimi-Othman, M., Nadzifah, Y., Umirah, N., & Ahmad, A. (2012). Toxicity of metals to tadpoles of the common Sunda toad, *Duttaphrynus melanostictus*. *Toxicological & Environmental Chemistry, 94*(2), 364–376. https://doi.org/10.1080/02772248.2011.640636

Singh, N. P., McCoy, M. T., Tice, R. C., & Schneider, E. L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research, 175*(1), 184–191. https://doi.org/10.1016/0014-4827(88)90265-0

Singha, U., Pandey, N., Boro, F., Giri, S., Giri, A., & Biswas, S. (2014). Sodium arsenite induced changes in survival, growth, metamorphosis and genotoxicity in the Indian cricket frog (*Rana limnocharis*). *Chemosphere, 112*, 333–339. https://doi.org/10.1016/j.chemosphere.2014.04.076

Sinley, J. R., Goettl, J. P., & Davies, P. H. (1974). The effects of zinc on rainbow trout (*Salmo gairdneri*) in hard and soft water. *Bulletin of Environmental Contamination and Toxicology, 12*, 193–201. https://doi.org/10.1007/BF01684960

Skidmore, J. (1964). Toxicity of zinc compounds to aquatic animals, with special reference to fish. *The Quarterly Review of Biology, 39*(3), 227–248. https://doi.org/10.1086/404229

Skordas, K., Kellepertzis, E., Kosmidis, D., Panagiotaki, P., & Vafidis, D. (2015). Assessment of nutrients and heavy metals in the surface sediments of the artificially lake water reservoir Karla, Thessaly, Greece. *Environmental Earth Sciences, 73*, 4483–4493. https://doi.org/10.1007/s12665-014-3756-1

Sliwinski, T., Czechowska, A., Kolodziejczak, M., Jajte, J., Wisniewska-Jarosinska, M., & Blasiak, J. (2009). Zinc salts differentially modulate DNA damage in normal and cancer cells. *Cell Biology International, 33*(4), 542–547. https://doi.org/10.1016/j.cellbi.2009.02.004

Stebler, E. F., Burks, S. L., Bantle, J. A., & Dawson, D. A. (1988). Evaluation of the developmental toxicity of metal-contaminated sediments using short term fathead minnow and frog embryo larvae assays. *Environmental Toxicology and Chemistry, 7*(1), 27–34. https://doi.org/10.1002/etc.5620070105

Stuart, S. N., Chanson, J. S., Cox, N. A., Young, B. E., Rodrigues, A. S., Fischman, D. L., & Waller, R. W. (2004). Status and trends of amphibian declines and extinctions worldwide. *Science, 306*(5702), 1783–1786. https://doi.org/10.1126/science.1103538

Sveccevičius, G. (1999). Fish avoidance response to heavy metals and their mixtures. *Acta Zoologica Lituanica, 9*(2), 103–113. https://doi.org/10.1080/13921657.1999.10512293

Tice, R. R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J. C.,...
A. Patar et al. Zinc contamination is an underestimated risk to amphibians: toxicity evaluation in tadpoles of...

World Health Organization. (2001). *International programme on chemical safety* (Environmental Health Criteria 221). Geneva.

Xie, H., Holmes, A. L., Young, J. L., Qin, Q., Joyce, K., Pelsue, S. C., Peng, C., Wise, S. S., Jeevarajan, A. S., Wallace, W. T., Hammond, D., & Sr, J. P. W. (2009). Zinc chromate induces chromosome instability and DNA double strand breaks in human lung cells. *Toxicology and Applied Pharmacology*, 234(3), 293–299. https://doi.org/10.1016/j.taap.2008.10.010

Yadav, S. S., Giri, S., Singha, U., Boro, F., & Giri, A. (2013). Toxic and genotoxic effects of Roundup on tadpoles of the Indian skittering frog (*Euflictis cyanophlyctis*) in the presence and absence of predator stress. *Aquatic Toxicology*, 132, 1–8. https://doi.org/10.1016/j.aquatox.2013.01.016

Yulin, W., Hongru, Z., & Lanying, H. (1990). Effects of heavy metals on embryos and larvae of flat fish *paralichthys olivaceus* [j]. *Oceanologia et Limnologia Sinica*, 21, 386–392.

Zhang, Y., Wang, Y., Yu, R., Zhang, S., & Wu, Z. (2008). Effects of heavy metals Cd$^{2+}$, Pb$^{2+}$ and Zn$^{2+}$ on DNA damage of loach *Misgurnus anguillicaudatus*. *Frontiers of Biology in China*, 3, 50–54. https://doi.org/10.1007/s11515-008-0012-3

& Sasaki, Y. (2000). Single cell gel/comet assay: Guidelines for in vitro and in vivo genetic toxicology testing. *Environmental and Molecular Mutagenesis*, 35(3), 206–221. https://doi.org/10.1002/(SICI)1098-2280(2000)35:3<206::AID-EM8>3.0.CO;2-J

Vilkina, G., Pomerantseva, M., & Ramalia, L. (1978). Absence of a mutagenic effect of cadmium and zinc salts in mouse somatic and sex cells. *Genetika*, 14, 2212–2214.

Vladimirov, V. (1969). Dependence of the embryonic development and viability of the carp on the trace element zinc. *Voprosy Ikhtiologii*, 9, 687–696.

Voroshilin, S., Plotko, E., Fink, T., & Nikiforova, V. (1978). Cytogenetic effect of inorganic compounds of tungsten, zinc, cadmium and cobalt on animal and human somatic cells. *Tsitologija i genetika*, 241–243.

Wei, B., & Yang, L. (2010). A review of heavy metal contaminations in urban soils, urban road dusts and agricultural soils from China. *Microchemical Journal*, 94(2), 99–107. https://doi.org/10.1016/j.microc.2009.09.014

Wei, L., Ding, G., Guo, S., Tong, M., Chen, W., Flanders, J., Shao, W., & Lin, Z. (2015). Toxic effects of three heavy metallic ions on *Rana zhenhaiensis* tadpoles. *Asian Herpetological Research*, 6(2), 132–142.