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

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**Highlights**

- $ZnCl_2$ produced concentration dependent mortality to *F. limnocharis* tadpoles.
- Sub-lethal $ZnCl_2$ altered metamorphosis time of tadpoles.
- $ZnCl_2$ 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 $ZnCl_2$ on *Fejervarya limnocharis* tadpoles under laboratory conditions. The $LC_{50}$ values of $ZnCl_2$ 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 $ZnCl_2$ induced significant mortality in concentration and time dependent manner. Sub-lethal $ZnCl_2$ exposure significantly altered survival, body length and body weight at metamorphosis. Micronucleus test and comet assay indicated the genotoxic potential of $ZnCl_2$. Significant increase in DNA strand break was observed following $ZnCl_2$ exposure equivalent to 1% of the of 24 h $LC_{50}$ 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 torphic 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

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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 polulation. 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 $ZnCl_2$ dissolved in distilled water was used as the test agent. $ZnCl_2$ (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 $LC_{50}$

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 $ZnCl_2$. 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 $LC_{50}$ values at different time points using probit analysis.

### 1.3. Chronic exposure and toxicity studies

Chronic toxicity evaluations were also made in polypropelyne tubs following sub-lethal $ZnCl_2$ 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 $ZnCl_2$ approximately ranging between 10% and 35% of the 24 h $LC_{50}$ 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 $ZnCl_2$ 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 $ZnCl_2$ (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 $LC_{50}$ value) of $ZnCl_2$. The use of this concentration is intended to determine the genotoxic potential of $ZnCl_2$ 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 $10^6$ 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 $Na_2EDTA$, 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 $Na_2EDTA$ 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 400×. Comet data was analyzed using 2-tailed Student's t-test.

## 2. Results

### 2.1. Acute toxicity studies and determination of $LC_{50}$

The acute $LC_{50}$ values of $ZnCl_2$ 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 $LC_{50}$ values decreased in a linear manner. Linear regression analysis of the mean lethal concentration showed significant ($R^2 = 0.851$, $p < 0.05$) concentration and time effect.

Table 1. $LC_{50}$ values of $ZnCl_2$ in *F. limnocharis* tadpoles

| Duration of exposure | $LC_{50}$ 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 $ZnCl_2$ 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, $ZnCl_2$ had significant ($p < 0.001$) effect as revealed by Kaplan–Meier product limit estimate. $ZnCl_2$ 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

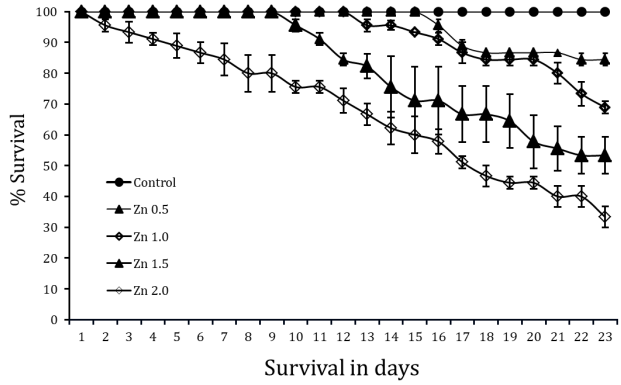


Figure 1. Survival of tadpoles of *F. limnocharis* after 23d of exposure to different sub-lethal concentrations of $ZnCl_2$. Values are mean ± SE

of $ZnCl_2$ 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 $ZnCl_2$ 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 $ZnCl_2$ 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.

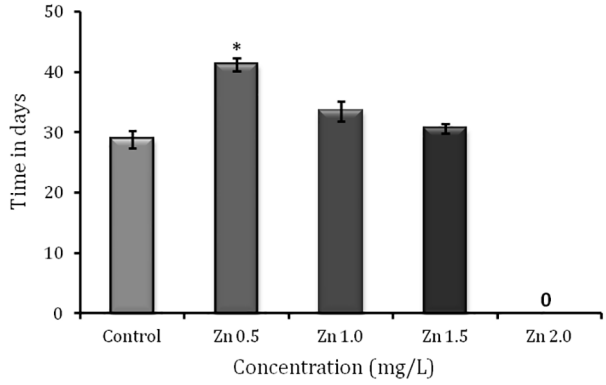


Figure 2. Time taken by tadpoles to metamorphose following exposure to different concentrations of $ZnCl_2$. Data are significantly different from control (ANOVA). (*) = $p < 0.05$

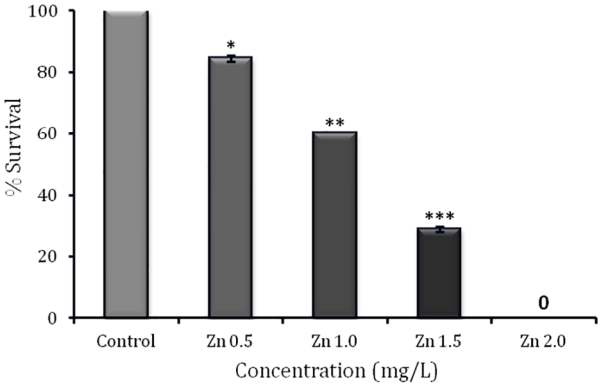


Figure 4. $ZnCl_2$ induced changes body weight (A) and snout to vent length (B) of froglets at metamorphosis. Data are significantly different from the control group at $p < 0.05$ (*)

Tadpoles exposed to highest concentrations of $ZnCl_2$ 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 $ZnCl_2$ (one-way ANOVA, $F_{4, 70}= 390.026$, $p < 0.001$).

The average body weight of the metamorphosed froglets in the $ZnCl_2$ 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 (Figre 4B). $ZnCl_2$ 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 $ZnCl_2$.

Table 2. Incidence of micronucleated erythrocytes induced by $ZnCl_2$ 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; CP: cyclophosphamide (positive control); [b] Values are frequency of micronucleated erythrocytes (%) expressed as means ± SE based on 1000 cells per animal (n = 15); [c] Level of significance from respective control values: *= $p < 0.05$: **= $p < 0.01$: ***= $p < 0.001$. Statistical analysis: ANOVA.

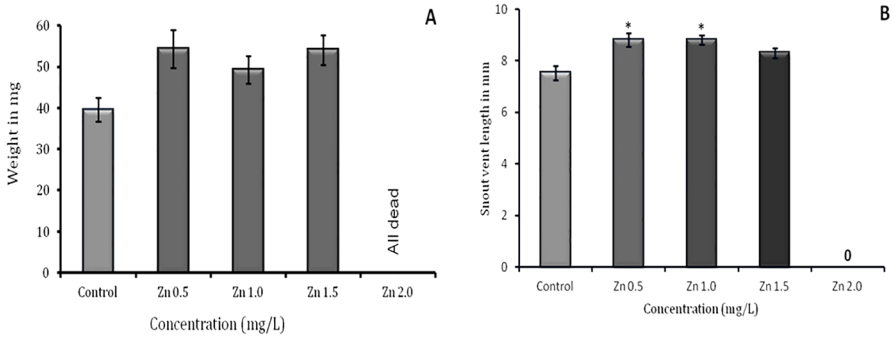


Figure 4. $ZnCl_2$ induced changes body weight (A) and snout to vent length (B) of froglets at metamorphosis. Data are significantly different from the control group at $p < 0.05$ (*)

### 2.3. Micronucleus test

$ZnCl_2$ exposure induced MN in the erythrocytes of *F. limnocharis* tadpoles (Table 2) at 24 h ($F_{4, 70} = 9.82$, $p < 0.05$), 48 h ($F_{4, 70} = 33.78$, $p < 0.01$), 72 h ($F_{4, 70} = 42.46$, $p < 0.001$), and 96 h ($F_{4, 70} = 47.53$, $p < 0.01$). There were significant positive correlations between the concentrations of $ZnCl_2$ 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 $ZnCl_2$ ($r = 0.6361$) all the other treatments tested showed time dependent increase in the frequency of MN all through the 96h study period. Moreover, it was found that that the overall time effect on micronucleus induction (ANOVA) was statistically significant ($F_{4, 295} = 4.82$, $p < 0.05$).

### 2.4. Comet analysis

Erythrocytes of *F. limnocharis* tadpoles showed significant change in the degree of DNA damage following $ZnCl_2$ exposure as evidenced by changes in comet parameters (Figure 6). Quantitative analysis revealed that amount of DNA

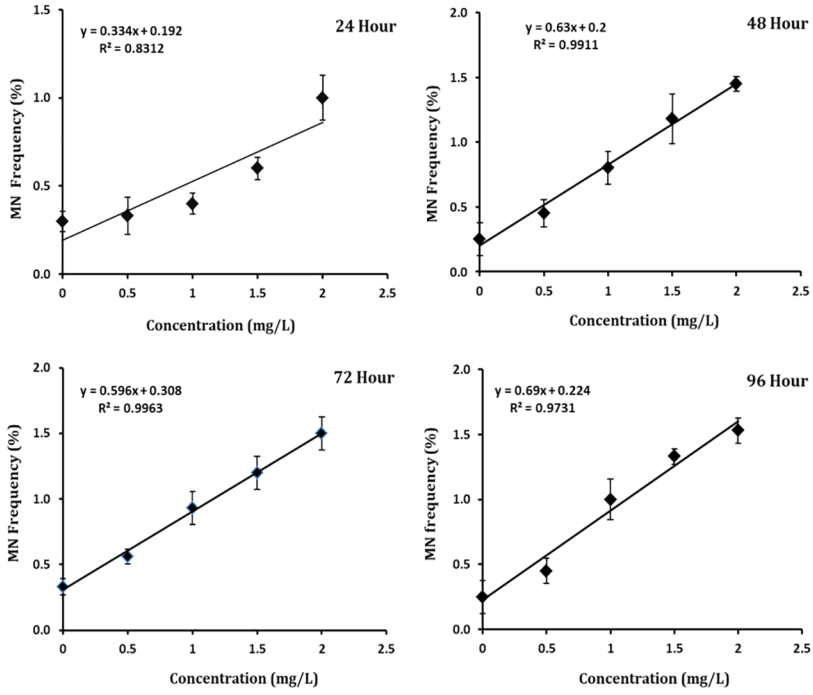


Figure 5. Regression plot and $R^2$ of micronucleated erythrocytes at 24 h, 48 h, 72 h and 96 h of zinc chloride treatment

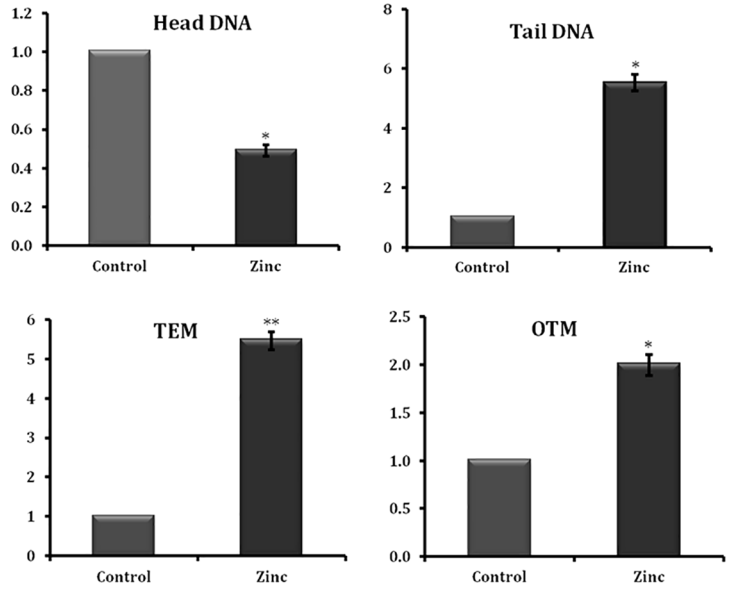


Figure 6. Comparison of comet parameters (fold change) between control and $ZnCl_2$ exposed groups. OTM: olive tail moment; TEM: tail extent moment. Values are significantly different from control: $p < 0.05$ (*) and $p < 0.001$ (**)

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 $ZnCl_2$ exposed cells was significantly higher ($p < 0.05$) compared to the control cells.

## 3. Discussion

The present study evaluated acute and subchronic toxicity of $ZnCl_2$ in tadpoles of *F. limnocharis*. It was found that tadpole mortality rate was positively correlated with exposure to $ZnCl_2$ concentration. The calculated 96 h $LC_{50}$ value of $ZnCl_2$ in the current study was found to be 3.61 mg/L (Table 1). Similar $LC_{50}$ value was also found in the earlier studies by Svecevičius (1999). Bagdonas and Vosylienė (2006) reported that the 96 h $LC_{50}$ value of zinc in Rainbow trout (*Oncorhynchus mykiss*) was 3.79 mg/L. The $LC_{50}$ values for $ZnCl_2$ 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 $LC_{50}$ values for zinc have been shown to vary over a wide range depending upon the species and developmental stages. For example, the 96 h $LC_{50}$ values was reported to be 2.1 mg/L for *Rana hexadactyla* and 28.38 mg/L for *Rana luteiventris* (Khangarot et al., 1985; Lefcort et al., 1998). However, available literature also reported that the most published $LC_{50}$ values for other amphibian tadpoles are greater than 19 mg/L (Linder & Grillitsch, 2000). One possible reason for this wide range of $LC_{50}$ data is due to the fact that toxicity of Zn ions is highly dependent on water hardness; the highest $LC_{50}$ value available in the literature was observed when concentration of calcium ions are at their highest (Skidmore, 1964). Moreover, other possible reason for this is due to the experimental methods conducted in each study such as body size or body length/developmental stage, body masses of tadpoles and temperature etc.

In the present study, long term exposure at sublethal concentrations (0.5–2.0 mg/L) of $ZnCl_2$ 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 $ZnCl_2$ concentration and the duration of exposure of the tadpoles. Tadpoles exposed to highest sub-lethal concentration of $ZnCl_2$ (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 $ZnCl_2$ 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 $ZnCl_2$ 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 environment 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, $ZnCl_2$ 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 $ZnCl_2$ (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. cyanophlyctis 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, $ZnCl_2$ 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 $ZnCl_2$ 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 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 vivo* (Vilkina et al., 1978). Kowalska-Wochna et al. (1988) reported that zinc chlorate 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 $ZnCl_2$ 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 $ZnCl_2$ 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.

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