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ORIGINAL ARTICLE

Toxic Effects of Three Heavy Metallic Ions on *Rana zhenhaiensis* Tadpoles

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Abstract Heavy metal pollution is widespread in some areas of China and results in contamination of land, water, and air with which all living organisms interact. In this study, we used three heavy metallic ions (Cu^{2+}, Pb^{2+} and Zn^{2+}) to assess their toxicity effects on mortality, blood biomarker and growth traits (body length and body mass) of *Rana zhenhaiensis* tadpoles. The results showed that the toxicity levels of the three metallic ions were different when conducted with different experiment designs. For acute toxicity tests, Cu^{2+} was the most toxic with the highest tadpole mortality. The mortalities of tadpoles showed significant differences among the treatments at the same exposure time endpoints (24, 48, 72 and 96h). Results from repeated measures ANOVA indicated that metallic ion concentration, exposure time and their interactions significantly affected the mortalities of *R. zhenhaiensis* tadpoles. Also, the toxicity effects of all binary combinations of the three metallic ion treatments showed synergism. The half lethal concentrations (LC_{50}) decreased with increasing exposure time during the experimental period, and the safe concentration (SC) values of Cu^{2+}, Pb^{2+} and Zn^{2+} were different from each other. Combined and compared LC_{50} values with previous data reported, it is suggestes that the toxicity levels of metal pollution to anuran tadpoles should be species-and age-related. For blood biomarker tests, Zn^{2+} was the most toxic with the highest total frequencies of abnormal erythrocytic nucleus. All three metallic ions caused higher abnormal erythrocytic nucleus compared with control groups. In a chronic toxicity test, Pb^{2+} was the most toxic with lowest growth traits. Survival rate (except for 18 days), total body length and body mass showed significant differences among the treatments. These findings indicated that tadpoles of *R. zhenhaiensis* should be as a bioindicator of heavy metals pollution.

Keywords Acute toxicity; micronucleus; chronic toxicity; growth; metal pollution; *Rana zhenhaiensis*

1. Introduction

The rapid and unprecedented decline of global biodiversity is of great concern and highlights the need to research the many different factors that can impact a species and its ecosystem. Amphibians play an important role in many ecological communities, ranging from helping nutrient cycles to serving as high quality prey items for predators, and as such their decline will impact on the ecosystems they are part of (deMaynadier and Hunter, 1995; Vertucci and Corn, 1996; Stuart *et al*., 2004; Natale *et al*., 2006; Hussain and Pandit, 2012).

Among amphibians, they have a biphasic life cycle comprising of an aquatic and terrestrial phase. They are highly sensitive to water pollution due to their association with aquatic habitats and permeable skin and are widely used in the monitoring of water contamination (Ezemonye and Tongo, 2009; Xia *et al*., 2012). Indeed, the pollution of anuran habitats is considered to be one of the major factors in their decline (Hussain and Pandit, 2012) with heavy metals considered as one of the worst chemical stressors due to their high toxicity at very low concentrations (Shuhaimi-Othma *et al*., 2012a). There have been many studies documenting the toxicity of exposure to metal compounds in different aquatic

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species. For example, in fish exposure metals such as copper (Cu^{2+}), cadmiun (Cd^{2+}) and Chromium (Cr^{6+}) is known to affect key parameters, including survival, growth and development and has been shown to interfere with the octavolateral system (Johnson *et al*., 2007). Freshwater insects, such as *Nais elinguis* were found to be more sensitive to exposure to Cu^{2+}, Cd^{2+}, iron (Fe^{3+}), manganese (Mn^{2+}), lead (Pb^{2+}), nickel (Ni^{2+}), zinc (Zn^{2+}) and aluminum (Al^{3+}) than freshwater worms (Shuhaimi-Othman *et al*., 2012b). In amphibians, identifying what effects being exposed to metals such as Cd^{2+}, Cu^{2+}, Pb^{2+} and Zn^{2+} have been carried out on a number of species including *Hypsiboas pulchellus* (Natale *et al.*, 2006), *Duttaphrynus melanostictus* (Shuhaimi-Othma *et al*., 2012a), *Bufo bufo gargarizans* (Yang and Jia, 2006), *Rana chensinensis* (Shi *et al*., 2007) and *R. catesbeiana* (Li and Tian, 2010). These studies show that although increases in metallic ion concentration and time of exposure leads to higher rates of mortality there is considerable species-specific variation in their sensitivity to different metallic ions. Moreover, in studies focusing on the sub-lethal/chronic effects of heavy metallic ions, such as *R. chensinensis* exposed to Pb^{2+} (Wang and Wang, 2008) and Cu^{2+} (Shi *et al*., 2007), *Pelophylax nigromaculatus* exposed to Pb^{2+}, Cu^{2+} and Hg^{2+} (Zhang, 2009; Huang *et al*., 2014) and *B. raddei* exposed to Cd^{2+} and Pb^{2+} (Zhang *et al*., 2007), found that individuals exhibited abnormal growth, development, behavior and erythrocytic nuclear abnormalities, which increased their susceptibility to predation and competition and overall decrease reproductive success.

Rana zhenhaiensis (previously *Rana japonica*) is common in Southeast China. This species is mainly found in rural areas with tadpoles living in low-lying, temporary water bodies and ditches (Zhou *et al*., 2005). Previous studies on this species have focused on their vulnerability to pesticides such as Triazophos (Zhong *et al*., 2011) and emamectin benzoate (Chen *et al*., 2011), and they found the tadpoles were highly sensitive to agricultural pesticides. In south and east China metals such as Cu, Cd, Zn, Pb, Cr, Fe are widely used in industry and are common water pollutants. As the full impact of these metallic ions on the aquatic habitats and species is still unknown. In this study, we examine the acute and chronic toxicity effects of three heavy metallic ions (Cu^{2+}, Pb^{2+} and Zn^{2+}) on tadpoles of *R. zhenhaiensis*, an important bio-indicator for water quality. The results of this work will provide a fundamental platform for establishing regulatory limits for metal loads in aquatic environments.

2. Materials and Methods

We collected Zhenhai brown frog (*R. zhenhaiensis*) eggs from a field in a suburb of Lishui City, Zhejiang Province, China, in March 2014. Eggs were then incubated within opaque plastic cages (60 cm length × 40 cm width × 30 cm height) with 20cm depth of dechlorinated tap water. Prior to experimentation the tadpoles were reared with commercial fish food (Shanghai Tech-bank feed industry Co. LTD). Tadpoles that were considered to be in good health (swimming freely, with good reflexes; average body weight = 0.04±0.001g; average body length = 1.54±0.11cm) were selected for toxicity treatment.

A standard stock solution of Cu^{2+}, Pb^{2+} and Zn^{2+} (100 mg/L) were prepared from analytical grade metallic salts of $CuSO_4{\cdot}5H_2O$, $ZnSO_4{\cdot}7H_2O$ and $(CH_3COO)_2Pb{\cdot}3H_2O$. The stock solutions were prepared with deinoized water in 1L volumetric flask and then kept for subsequent concentration dilutions.

2.1 Pre-experiment A wide range of metal solution concentrations were used in the pre-experiment; seven Cu^{2+} (0.2, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/L), five Pb^{2+} (10.0, 30.0, 50.0, 70.0 and 90.0 mg/L) and six Zn^{2+} (5.0, 10.0, 20.0, 30.0, 40.0 and 50.0 mg/L). Each metal-treated concentration group consisted of 2 replicates of 10 randomly allocated tadpoles in a round 1000mL plastic container with 500 mL of the appropriate solution. The numbers of dead tadpoles in each container were counted 48h later. By observing tadpole mortality the lethal concentration of no mortality (LC_0) and maximum mortality (LC_{100}) were used to obtain the range concentration of LC_0 to LC_{100} for the following experiments (Wei *et al*., 2014).

2.2 Acute toxicity Based on the pre-experiment results static-water tests were used in the toxicity experiments (Zhou and Zhang, 1989). Six Cu^{2+} (0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 mg/L), five Pb^{2+} (30.0, 40.0, 50.0, 60.0 and 70.0 mg/L) and Zn^{2+} (10.0, 20.0, 30.0, 35.0 and 45.0 mg/L) were chosen for the acute toxicity experiments with each having their own control (0.0 mg/L). 15 experimental tadpoles were randomly allocated to each metal-treated concentration in a 1000 mL plastic container with 800 mL metal solution. Each concentration treatment was conducted in triplicate at room condition. Mortality was recorded every 24 hours for 4 days (96 hours) for each treatment. Tadpoles were recorded as dead when they turned upside down and sank to the bottom of the container or when their tail showed no form of movement even when prodded with a glass rod (Mgbaeruhu, 2002).

During the acute toxicity test, the tadpoles were not fed.

2.3 Joint toxicity To examine the joint toxicity of the three metallic ions, pairwise combinations were performed based on the results of the acute toxicity tests. The half lethal concentration (LC_{50}) acute toxicity test at 48h was then taken as 1 toxic unit (U). For the following joint toxicity testing, test concentrations of the three metallic ions combined are listed in table 1. Ten experimental tadpoles were randomly allocated to each joint concentration following the method of acute toxicity tests. The mortalities of tadpoles exposed to combined metals at 48h were recorded following the methods of Chen *et al*. (2007).

2.4 Blood biomarkers To further investigate the toxic effects of the three metallic ions on *R. zhenhaiensis* tadpoles, blood parameter measurements were conducted. Five tadpoles were randomly exposed to three new concentrations of each metallic ion (0.10, 0.20 and 0.33 mg/L) with 4 replications respectively over a period of 4 days (96h). One more treatment (0 mg/L) was set up for control. Every 24 hours genotoxicity of each treatment was tested using the measuring erythrocytic nuclear assay (ENA), carried out in mature peripheral erythrocytes according to the procedures of Guilherme *et al* (2008). The blood smear of the live tadpoles were fixed with methanol for 10 min and stained with 10% Giemsa for 15–20 min. For each smear, 500 erythrocytes were observed and scored under 1000× magnification to determine the frequency of the following nuclear lesions categories: mitotic (M), binucleated (BN), micronuclei (MN), 8 shape nuclei (8SN), karyopyknosis (K), anucleated (AN) and unequal division (UD). The control group was only carried out after the 48h exposed. The results were expressed as the mean value (%) of the sum (M+BN+MN+8SN+K+AN+UD) for all the lesions observed (Guilherme *et al*., 2008).

2.5 Chronic toxicity Chronic toxicity tests were carried out in a similar manner as the acute tests. Only one low concentration (1/10 toxic U) of each metallic ion was used. Thus the treatment concentrations of Cu^{2+}, Pb^{2+} and Zn^{2+} were 0.055, 4.44 and 2.40 mg/L, respectively. Tests were done using three replications per metallic ion and one control group. Ten tadpoles were randomly allocated to each container. Exposure lasted for 18 days, and growth traits including survival rate, body mass and total body length (the length from snout to tail tip) of tadpoles were collected every 6 days. Tadpoles were reared with commercial fish food (Shanghai Tech-bank feed industry Co. LTD). A new stock solution for each metallic ion was made up every 3 days immediately before each water change.

2.6 Data analysis Prior to any statistical tests all variables were tested for normality and homogeneity. For the acute tests, One-way ANOVA and Tukey's post hoc multiple comparisons test were used to evaluate the effects of each metal on the mortalities of tadpoles under different concentrations and different exposure times. To examine the correlated effects of both concentration and exposure on tadpole mortality repeated measures ANOVA was used. For comparisons of the growth data among the three metallic ions in the chronic toxicity tests, One-way ANOVAs were mainly used. Statistical analyses were conducted via Statistica 6.0, with α=0.05 taken as statistically significant.

Half lethal concentration (LC50) for each metallic ion was determined using probit analyses and straight line interpolations (Chen *et al*., 2007), while the corresponding safe concentrations (SC) were carried out with two typical equations:

SC I = $(48h\text{-}LC_{50}\times 0.3)/(24h\text{-}LC_{50}/48h\text{-}LC_{50})^2$ (Zhang *et al*., 2011)

SC II = $96h\text{-}LC_{50}\times 0.1$ (Ezemonye and Tongo, 2009)

The evaluation of joint toxicity for each binary metallic ions combined was conducted using characteristic of mortality-concentration curves based on data recorded at 48h exposure. When the mortality was > 50%, the co-effects were taken as synergistic; in turn, when the mortality < 50%, the coeffects were taken as antagonistic (Chen *et al*., 2007).

3. Results

3.1 Acute toxicity and joint toxicity When *R. zhenhaiensis* tadpoles were exposed to the three different metallic ion solutions over the same exposure

Table 1 The proportion of binary combined concentrations of the three metallic ions.

ions combined	concentration (mg/L)				
	0.2U + 0.8U	0.4U + 0.6U	0.5U + 0.5U	0.6U + 0.4U	0.8U + 0.2U
Cu-Pb	0.11+35.51	0.22+26.63	0.28+22.20	0.33+17.76	0.44+8.88
Zn-Pb	4.80+35.51	9.60+26.63	12.00+22.20	14.39+17.76	19.20+8.88
Cu-Zn	0.11+19.20	0.22+14.39	0.28+12.00	0.33+9.60	0.44+4.80

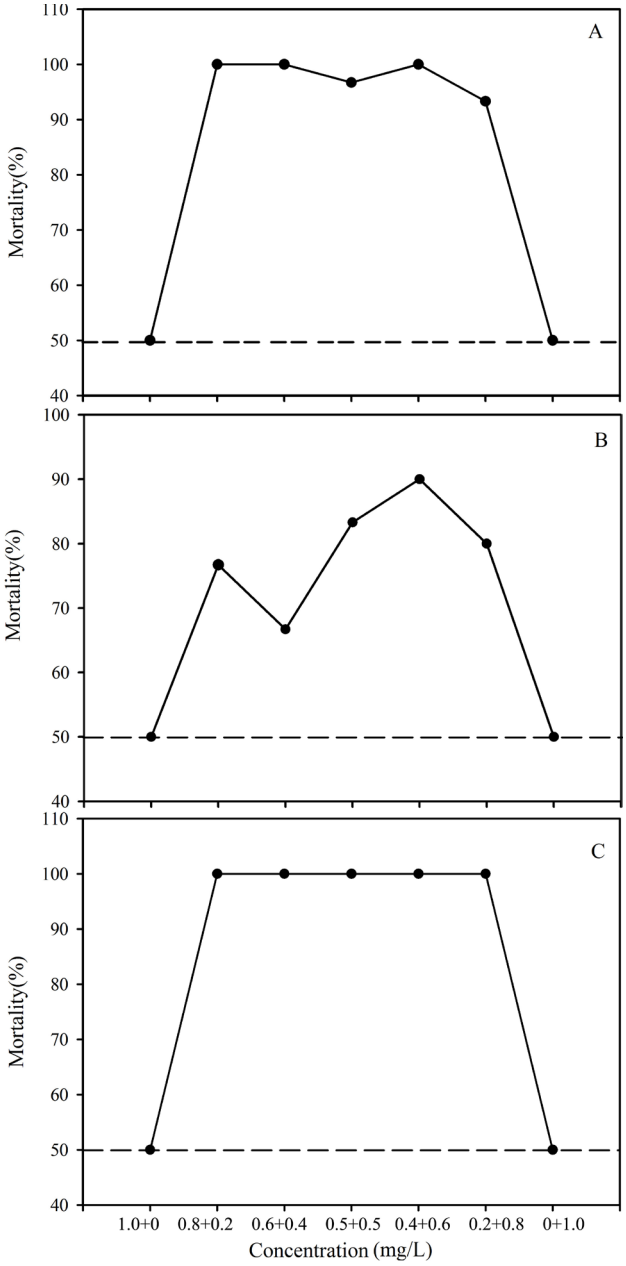

Figure 1 Joint toxicity effectes of the three metallic ions binary combined, Cu-Pb (A), Zn-Pb (B) and Cu-Zn (C).

times tadpole mortality was significantly different between treatments (P<0.001, Table 2) with half lethal concentrations (LC_{50}) decreasing with increasing exposure time. Variation between metallic ion types was also observed in the safe concentration (SC) values (Table 3) with overall toxicity levels going from Cu^{2+}> Zn^{2+}> Pb^{2+}. The results of repeated measures ANOVA indicated that metallic ion concentration and exposure time significantly affected *R. zhenhaiensis* tadpole mortality (Table 4).

For pairwise ions combined tests, the toxicity effects of all the three treatment groups were similar (Figure 1). However, tadpole mortality was not significant different between the various concentrations within each joint treatments (Cu-Pb: $F_{4,10}$=2.000, P=0.171; Zn-Pb: $F_{4,10}$=0.657, P=0.635; Cu-Pb: $F_{4,10}$=1.000, P=0.452, One-way ANOVA).

3.2 Blood biomarker All tadpoles in 0.33 mg/L of Cu^{2+} treatment died before the end of their 12 hour exposure. Overall the three metallic ions produced seven different types of erythrocyte abnormalities: mitotic, binucleated, micronuclei, 8 shape nuclei, karyopyknosis, anucleated and unequal division (Figure 2). The results showed that the total frequencies of abnormal erythrocytic nuclei (TFAEN) were all significantly higher than the control group (Table 5). Moreover, during the same exposure concentration, frequencies of abnormal erythrocytic nuclei observed (FAEN) were also different among various exposure times (Table 5).

3.3 Chronic toxicity Tadpoles exposed to the three metallic ions at low concentrations showed differences in growth pattern compared to the control group (Figure 3). The percentage survival of the exposed tadpoles was reduced compared to that of the control group, and significant differences were found at day 6 ($F_{3,11}$=9.200, P=0.006, One-way ANOVA) and at day 12 ($F_{3,11}$=4.133, P=0.048, One-way ANOVA) but not on day 18 (Figure 3A). The Pb^{2+} treatment recorded the lowest survival rate, body mass and smallest total body length across all time periods. Cu^{2+} treatment caused significantly lower body lengths across all time periods compared to Zn^{2+} treatment and also when compared to the control for body mass and body length (all $P < 0.05$, One-way ANOVA, Figure 3B and 3C).

4. Discussion

4.1 Acute and joint toxicity Our study shows that tadpole mortality was positively correlated with heavy metallic ion concentration. However, mortality rates were not uniform across the different metal types with copper being more toxic than zinc which was more toxic than lead (Cu^{2+} > Zn^{2+} > Pb^{2+}; Table 2). These results correspond with previous studies by Khangarot *et al.* (1985) and Yang and Jia (2006) who identified the level of toxicity of different metallic ions on *R. hexadactyla* and *B. bufo gargarizans* tadpoles to be Cu^{2+} > Zn^{2+} > Fe^{3+} > Pb^{2+} and Cu^{2+} > Cd^{2+} > Zn^{2+}, respectively. In contrast, Shuhaimi-Othman *et al.* (2012b) found that *Duttaphrynus melanostictus* tadpoles were more sensitive to Cu^{2+} > Cd^{2+} > Fe^{3+} > Al^{3+} > Pb^{2+} > Zn^{2+} > Ni^{2+} > Mn^{2+} indicating

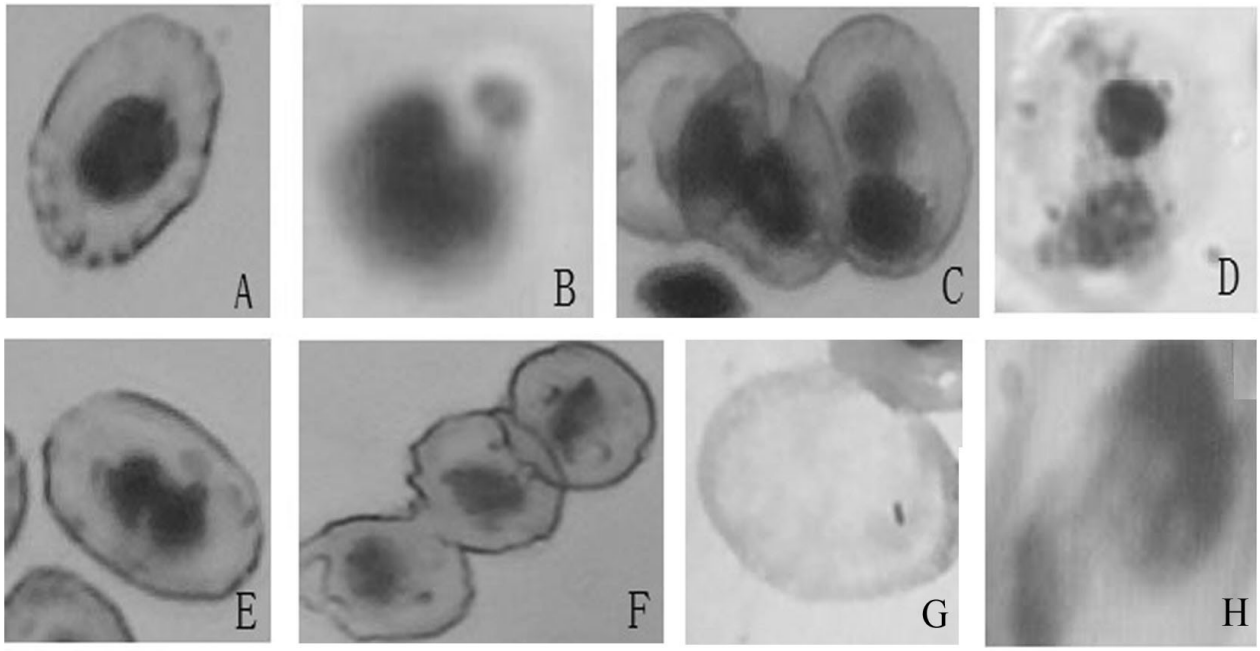

Figure 2 The three metallic ions treatments produced different abnormalities in erythrocyte: Normal cell (A), mitotic (B), binucleated (C), micronuclei (D), 8 shape nuclei (E), karyopyknosis (F), anucleated (G) and unequal division (H).

Table 2 The acute toxicity test results of the three metallic ions alone to *R. zhenhaiensis* tadpoles (n = 15).

Concentration(mg/L)	mortality (%)			
	24h	48h	72h	96h
Cu^{2+}				
0.3	2.2^{d}	2.2^{c}	11.1^{d}	11.1^{c}
0.4	2.2^{d}	8.9^{c}	24.4^{d}	48.9^{b}
0.5	17.8^{cd}	35.5^{b}	42.2^{c}	73.3^{ab}
0.6	48.9^{bc}	86.7^{a}	91.1^{a}	93.3^{a}
0.7	71.1^{ab}	80^{a}	82.2^{b}	95.6^{a}
0.8	91.1^{a}	95.6^{a}	100^{a}	100^{a}
One-way ANOVA	$F_{5,17}$ = 29.842**	$F_{5,17}$ = 108.558**	$F_{5,17}$ = 155.055**	$F_{5,17}$ = 28.690**
Pb^{2+}				
30	0^{c}	6.7^{c}	15.6^{b}	37.8^{b}
40	8.9^{bc}	37.8^{bc}	82.2^{a}	91.1^{a}
50	24.4^{ab}	64.4^{ab}	82.2^{a}	88.9^{a}
60	22.2^{ab}	82.2^{a}	97.7^{a}	97.7^{a}
70	33.3^{a}	100^{a}	100^{a}	100^{a}
One-way ANOVA	$F_{4,14}$ = 14.792**	$F_{4,14}$ = 17.641**	$F_{4,14}$ = 63.684**	$F_{4,14}$ = 26.860**
Zn^{2+}				
10	0^{d}	0^{c}	6.7^{c}	31.1^{b}
20	4.4^{cd}	75.6^{b}	77.8^{b}	86.7^{a}
30	53.3^{abc}	95.6^{a}	97.8^{a}	97.8^{a}
35	51.1^{bc}	100^{a}	100^{a}	100^{a}
45	97.8^{a}	100^{a}	100^{a}	100^{a}
One-way ANOVA	$F_{4,14}$ = 17.021**	$F_{4,14}$ = 133.577**	$F_{4,14}$ = 203.625**	$F_{4,14}$ = 80.591**

Note: ** indicated significant differences at $P < 0.001$. Types with different superscripts differ significantly (Tukey's test, $\alpha = 0.05$, a > b > c > d).

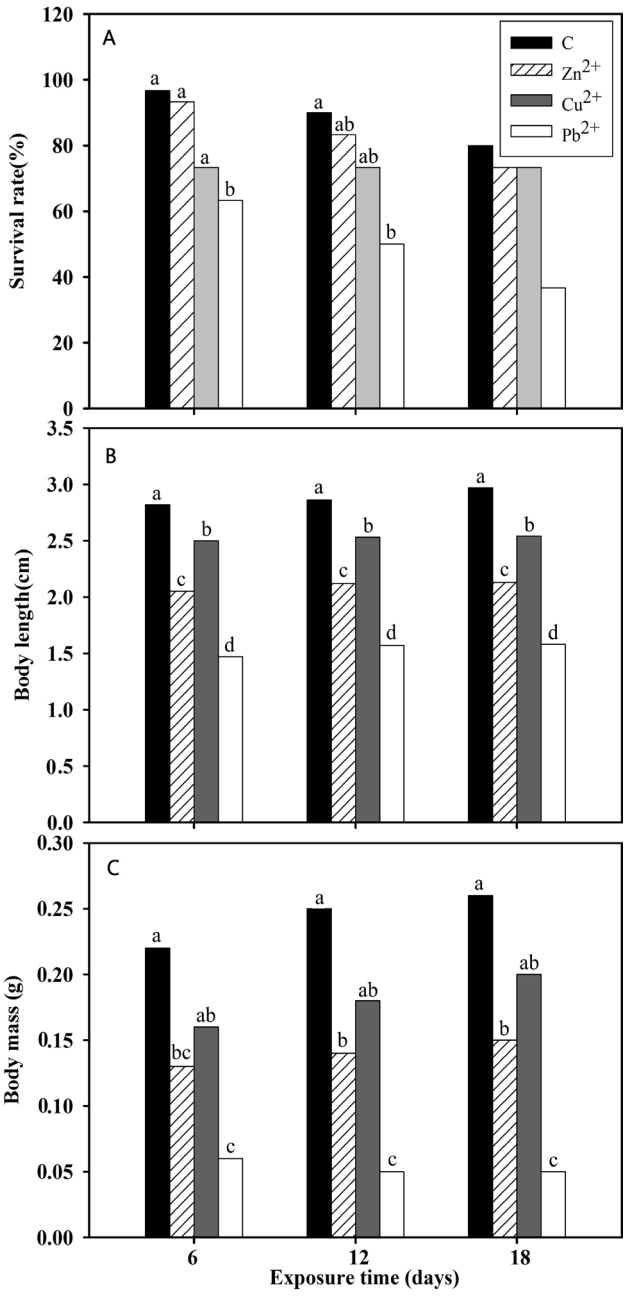

Figure 3 Comparisons of survival rate (A), mean total body length (B) and mean body mass (C) of *R. zhenhaiensis* exposed to relevant concentrations of the three metallic ions with those of the control group. Types with different superscripts differ significantly (Tukey's test, $\alpha = 0.05$, a > b > c > d).

that some species are more susceptible to certain metallic elements more than others.

This study showed that LC_{50} values for 24, 48, 72 and 96h of Cu^{2+}, Pb^{2+} and Zn^{2+} were 0.62, 67.08 and 32.90; 0.55, 44.39 and 23.98; 0.48, 31.72 and 16.88, and 0.43, 31.72 and 12.21 mg/L, respectively (Table 3). Compared to other studies that have investigated the toxicity of the three heavy metallic ions on anuran species at their different life stages, especially for tadpoles, we found different LC_{50} values produced by different study species (Table 3, Table 6). One possible reason for this is because the experimental methods conducted in each study, such as body size/developmental stage, body mass of tadpoles and experimental water (soft or hard) were different. For example, Rao and Madhyastha (1987) conducted a study on the toxicity of heavy metallic ions (Hg^{2+}, Cd^{2+}, Cu^{2+}, Mn^{2+}, and Zn^{2+}) on different ages (1- and 4-week old) of tadpoles of *M. ornata* and found that 4-week old tadpoles were more sensitive toward heavy metallic ions than were l-week old ones. Also, Harris *et al.* (2000) conducted toxicity testing with *R. pipiens* and *B. americanus* to pesticides, and found that the former was more sensitive to pesticides than the latter. This is probably due to species and age related differences in susceptibility to pesticides and heavy metallic ions.

In this study we found that Cu^{2+} was approximately 100 times more toxic to *R. zhenhaiensis* tadpoles than Pb^{2+} and 50 times more toxic than Zn^{2+}. Typically, aquatic organisms sensitivity to trace metals follows the trend: $Hg^{2+} > Ag^{+} > Cu^{2+} > Cd^{2+} > Zn^{2+} > Ni^{2+} > Pb^{2+} > Cr^{6+} > Sn^{4+}$ (Luoma and Rainbow, 2008). However, some toxicity studies with other species found that Pb^{2+} was more toxic than Zn^{2+}, such as with *D. melanostictus* (Shuhaimi-Othman *et al*., 2012a), *R. hexadactyla* (Khangarot *et al*., 1985), *Nais slinguis* (Shuhaimi-Othman *et al*., 2012b). Therefore, It is by no means the case that all essential metals are more toxic than all nonessential metals.

Comparisons of the pairwise joint toxicity treatments were highly similar across treatments (Figure 1). This corresponds to a study on *R. limnocharis* tadpoles by Jia *et al.* (2005) who found similar results when testing the joint toxicity of Cu-Zn. Compared to previous studies reporting on the toxicity of metallic ions to aquatic organisms, we found that different results produced by different species. For example, the joint toxicity of Cu-Zn conducted by Yang and Jia (2006) with *B. bufo gargarizans* showed antagonistic. Likewise, the joint toxicity of Cu-Zn, Pb-Zn, and Cu-Pb conducted by Chen *et al.* (2007) with *Hydra sp*, and Pb-Zn conducted by Zhang *et al.* (2011) with *Carassius auratus* showed antagonistic. This is probably because the co-effects of metallic ions are complicated, which the way they act on cells are different (Eaton, 1973).

4.2 Blood biomarker The toxic effects of Cu^{2+}, Pb^{2+} and Zn^{2+} on the tadpoles could be observed in their blood tests (Table 5). The total frequency of abnormal erythrocytic nuclei (TFAEN) increased by increasing exposure concentration for all the three metallic ions (Table 5).

Table 3 The half lethal concentrations (LC_{50}) and safe concentrations (SC) of the three metallic ions, mg/L.

Metal	Exposure time	Simulation equation	R^2	LC_{50} (mg/L)	SC I	SC II
Cu^{2+}	24h	$Y = 7.260x + 0.494$	0.970*	0.62	0.130	0.043
	48h	$Y = 7.615x + 0.800$	0.933*	0.55		
	72h	$Y = 8.881x + 0.762$	0.863*	0.48		
	96h	$Y = 8.794x + 1.213$	0.949*	0.43		
Pb^{2+}	24h	$Y = 0.097x–1.507$	0.651	67.08	5.832	3.172
	48h	$Y = 0.117x–0.194$	0. 901*	44.39		
	72h	$Y = 0.105x + 1.044$	0.927*	37.68		
	96h	$Y = 0.087x + 2.240$	0.891*	31.72		
Zn^{2+}	24h	$Y = 0.189x–1.219$	0.943*	32.90	3.822	1.221
	48h	$Y = 0.243x–0.826$	0.837*	23.98		
	72h	$Y = 0.156x + 2.366$	0.922*	16.88		
	96h	$Y = 0.128x + 3.437$	0.924*	12.21		

Note: *indicated significant correlated, $P < 0.05$.

Table 4 The effects of ion concentration, exposure time and their interactions on mortality of *R. zhenhaiensis* tadpoles.

Types of ion	Concentration	Exposure time	Concentration×Exposure time
Cu^{2+}	$F_{5,12} = 76.735^{**}$ I^c, II^c, III^b, IV^a, V^a, VI^a	$F_{3,36} = 72.267^{**}$ $24h^d$, $48h^c$, $72h^b$, $96h^a$	$F_{10,36} = 6.881^{**}$
Pb^{2+}	$F_{4,10} = 42.066^{**}$ I^c, II^b, III^{ab}, IV^a, V^a	$F_{3,30} = 196.\ 715^{**}$ $24h^c$, $48h^b$, $72h^a$, $96h^a$	$F_{12,30} = 8.578^{**}$
Zn^{2+}	$F_{4,10} = 113.240^{**}$ I^c, II^b, III^a, IV^a, V^a	$F_{3,30} = 65.559^{**}$ $24h^b$, $48h^a$, $72h^a$, $96h^a$	$F_{12,30} = 9.207^{**}$

Note: ** indicated significant differences at $P < 0.001$. Types with different superscripts differ significantly (Tukey's test, $\alpha = 0.05$, a > b > c > d), I, II, III, IV and V indicated different metallic ion concentrations, respectively.

Zn^{2+} was the most toxic to the tadpoles blood red cells followed by Pb^{2+} and then Cu^{2+} at 0.10 mg/L treatments, Zn^{2+}>Cu^{2+}>Pb^{2+} at 0.20 mg/L treatments and Zn^{2+}>Pb^{2+} with 0.33 mg/L treatment (Table 5). This is in agreement with the results of Rosenberg *et al*. (1998) conducted with *B. arenarum* exposed to Pb^{2+} and Jiang *et al.* (2008) conducted with *B. melanostictus* exposed to Cu^{2+}, Pb^{2+} and Hg^{2+}, which showed that there was a micronuclei response to heavy metallic ions. Interestingly, Jiang *et al.* (2008) and Zhang (2009) also found that TFAEN produced by *B. melanostictus* and *Pelophylax nigromaculatus* were higher in Pb^{2+} treatments than in Cu^{2+} treatments. However, other micronuclei studies with other species found that Cu^{2+} was more toxic than Pb^{2+}, such as *B. gargarizans* (Zhou *et al*., 2008). Although these contrasting results may be due to different methodologies, they also show that amphibian hematological parameters will be affected by the interactions between various combinations of nutrients, metals and pesticides (Ilizaliturri-Hernandez *et al*., 2013). Thus, erythrocytic nuclear abnormalities (ENA) should be recognized as one type of biomarker to assess water quality and the genotoxicity of contaminants on organisms (Costa *et al*., 2011).

4.3 Chronic toxicity This study revealed the chronic toxicity of Cu^{2+}, Pb^{2+} and Zn^{2+} to *R. zhenhaiensis* tadpoles over an 18-day period. The tadpoles were exposed to 1/10 LC_{50} concentration of metallic ions with the toxic effects were recorded over several time points. The results showed that the three metallic ions affected the growth of the tadpoles compared to the control group. In the Pb^{2+} treatment survival rate, body length and body mass were all lower than those in Cu^{2+} and Zn^{2+} treatments (Figure 3) showing that Pb^{2+} was the most toxic to the development of *R. zhenhaiensis* tadpoles. Similar results were seen in a study by Jiang *et al* (2008). on *B. melanostictus*, although Jackson *et al*. (2005) found that Pb^{2+} was less toxic than other metallic ions in *Callianassa kraussi*.

In conclusion, we predict that Cu^{2+}, Pb^{2+} and Zn^{2+} could significantly affect the mortality, blood biomarker and growth traits of *R. zhenhaiensis* tadpoles. It is indicated

Table 5 Effects of the three metallic ions on micronuclei of red blood cells of *R. zhenhaiensis* tadpoles. *N* = the numbers of trial tadpoles, *n* = erythrocytic cells from smear observed, TNAEN = total numbers of abnormal erythrocytic nucleus observed, FAEN = frequency of abnormal erythrocytic nucleus observed, TFAEN = total frequency of abnormal erythrocytic nucleus observed

Ions	Concentration/mgL^{-1}	Time	*N*	*n*	TNAEN	FAEN/‰	TFAEN/‰
Cu^{2+}	0.10	24h	5	2500	35	14.0	16.0
		48h	5	2500	42	16.8	
		72h	5	2500	43	17.2	
		96h	5	2500	40	16.0	
	0.20	24h	5	2500	53	21.2	26.6
		48h	5	2500	70	28.0	
		72h	5	2500	70	28.0	
		96h	5	2500	73	29.2	
Pb^{2+}	0.10	24h	5	2500	24	9.6	19.1
		48h	5	2500	44	17.6	
		72h	5	2500	49	19.6	
		96h	5	2500	74	29.6	
	0.20	24h	5	2500	55	22.0	25.9
		48h	5	2500	76	30.4	
		72h	4	2000	46	23.0	
		96h	4	2000	56	28.0	
	0.33	24h	4	2000	37	18.5	30.1
		48h	4	2000	44	22.0	
		72h	3	1500	37	24.7	
		96h	3	1500	93	62.0	
Zn^{2+}	0.10	24h	5	2500	41	16.4	30.1
		48h	5	2500	71	28.4	
		72h	5	2500	90	36.0	
		96h	5	2500	99	39.6	
	0.20	24h	5	2500	51	20.4	32.6
		48h	5	2500	72	28.8	
		72h	5	2500	100	40.0	
		96h	5	2500	103	41.2	
	0.33	24h	5	2500	56	22.4	37.9
		48h	5	2500	83	33.2	
		72h	5	2500	112	44.8	
		96h	5	2500	128	51.2	
Control	0	48h	5	2500	12	4.8	4.8

that different heavy metallic ions should produced various toxic effects to organisms. Therefore, *R. zhenhaiensis* tadpoles should be a potential objective in toxicity testing and as a bioindicator of heavy metals pollution.

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Table 6 Comparisons of LC_{50} values of different larval amphibian species tadpoles tested with the three heavy metallic ions.

Irons	Species	Body length/cm	Body mass/g	Exposure time	LC_{50} (mg/L)	References
Cu^{2+}	*Bufo bufo gargarizans*	1.05	0.16	48h	0.514	Yang and Jia, 2006
				72h	0.474	
				96h	0.288	
	Rana chensinensis	0.567	0.046	24h	0.131	Shi *et al.*, 2007
				48h	0.105	
				72h	0.038	
	Rana limnocharis	1.18	0.25	24h	0.98	Jia *et al.*, 2005
				48h	0.70	
				96h	0.43	
	Rana nigromaculata	1.04	0.011	24h	1.98	Wang *et al.*, 2001
				48h	1.66	
	Duttaphrynus melanostictus	2.0-2.5	0.11	24h	0.08	Shuhaimi-Othman *et al.*, 2012a
				48h	0.05	
				72h	0.04	
				96h	0.03	
	Rana catesbeiana	4.0	1.0	12h	0.51	Li and Tian, 2010
				24h	0.46	
				48h	0.31	
				72h	0.24	
Pb^{2+}	*Rana nigromaculata*	1.04	0.011	24h	30.45	Wang *et al.*, 2001
				48h	30.18	
	Rana chensinensis	2.7	0.3	24h	3.30	Wang and Wang, 2008
				48h	1.66	
				72h	1.41	
	Duttaphrynus melanostictus	2.0-2.5	0.11	24h	8.2	Shuhaimi-Othman *et al.*, 2012a
				48h	3.5	
				72h	2.2	
				96h	1.5	
	Rana catesbeiana	4.0	1.0	12h	38.7	Li and Tian, 2010
				24h	35.1	
				48h	33.4	
				72h	31.9	
Zn^{2+}	*Duttaphrynus melanostictus*	2.0-2.5	0.11	24h	18.0	Shuhaimi-Othman *et al.*, 2012a
				48h	14.0	
				72h	9.2	
				96h	4.2	
	Bufo bufo gargarizans	1.05	0.16	48h	33.56	Yang and Jia, 2006
				72h	31.25	
				96h	30.76	
	Rana limnocharis	1.18	0.25	24h	60.0	Jia *et al.*, 2005
				48h	51.0	
				96h	46.5	

assistance with laboratory work.

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