

Toxicity of Arsenic, Aluminum, Chromium and Nickel to the Embryos of the Freshwater Snail, *Radix quadrasi* von Möellendorf 1898

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Heavy metals are one of the major toxicants affecting different organisms including freshwater snails. To examine this problem, the effects of arsenic (As^{3+}), aluminum (Al^{3+}), chromium (Cr^{6+}), and nickel (Ni^{2+}) on the embryonic development of the freshwater pulmonate, *Radix quadrasi*, were determined. For the acute toxicity of individual metals, a total of 100 egg masses were subjected to static renewal test for 96h. Sublethal toxicity test based on adjusted LC_{10} -96h concentrations was conducted for 14 days. Based on the LC_{50} -96h, the toxicity trend for embryos was Cr^{6+} (0.0263 mg/L) > As^{3+} (1.0147 mg/L) > Ni^{2+} (1.5877 mg/L) > Al^{3+} (1.8787 mg/L). Sublethal toxicity test showed growth retardation as the most common abnormality among embryos exposed to As^{3+} , Al^{3+} and Ni^{2+} , followed by edema and thinning of the shell. The hatchability and incubation period were also significantly decreased and prolonged in all treatment groups as compared to the control. The lowest observed effective concentration which induced abnormality was lower than the criteria continuous concentration and comparable to the detected field levels in polluted Philippine freshwater system. The present study demonstrated *R. quadrasi* embryos as a general sensitive bioindicator for trace levels of As^{3+} , Al^{3+} , Ni^{2+} , and Cr^{6+} .

Key Words: embryos, freshwater snail, heavy metals, pollution, toxicity

INTRODUCTION

Persistence of heavy metals in aquatic ecosystem is one of the major environmental concerns in developing countries as they cause adverse effects on the health and reproductive capacity of different organisms including many invertebrates. Waterborne heavy metals such as arsenic prolonged hatching and decreased the fecundity in *Biomphalaria glabrata* (Ansaldo et al. 2009), while aluminum altered the feeding activity in *Lymnaea stagnalis* (Elangovan et al. 2000). Chromium increased the mortality of *Artemia salina* and inhibited the naupliar development of *Mesocyclops pehpeiensis* (Wong & Pak 2004) while nickel reduced the hatching success and compromised larval development in *Atherinops affinis*, *Haliotis rufescens*, and *Mysidopsis intii* (Hunt et al. 2002). At present, metals and metalloids from agricultural,

domestic, and industrial activities are often discharged excessively into rivers and lakes. Thus, assessment of their toxicity threshold has since become an important component of water pollution monitoring (Canivet et al. 2001; Ansaldo et al. 2009).

In the Philippines, heavy metal contamination is attributed to improper waste disposal, mine tailing spills, and chemical run-offs (Greenpeace 2007). In Laguna Lake, sediment analysis revealed that copper was the heavy metal with the highest concentration (86.9 to 116.9 mg/kg) followed by lead (17.0-23.0 mg/kg), chromium (13.2-27.1 mg/kg), zinc (10.6-18.3 mg/kg), nickel (9.7-18.7 mg/kg), and cadmium (0.02-0.09 mg/kg) (Hallare et al. 2005). Chavez et al. (2006) also detected dissolved trace levels of arsenic (0.0017-0.0070 mg/kg) and mercury (0.0157-0.0050 mg/kg) in the lake. Mandal and Suzuki (2002) reported 0.1 mg/L As^{3+} in Matingao and Marbol rivers in Mindanao. David (2003), on the other hand, found residual concentration of

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aluminum (0.005-8.71 mg/kg) in Boac-Makulapnit River in Marinduque. Despite the detection of these metals, however, limited studies have explored the effects of these field concentrations on native aquatic organisms.

Metal toxicity in freshwater ecosystem is very pervasive because metal ions form complexes with sulfhydryl groups in proteins and smaller biological molecules to a greater extent (Spehar & Fiandt 1986; Foulkes 2000). This affinity to biological systems results in the vulnerability of various resident biota (Hunt et al. 2002; Liao et al. 2004). Among the most affected aquatic animals are pulmonate snails because they move very slowly and could not easily escape polluted habitats. They also accumulate heavy metals more in their tissue inducing numerous acute and sublethal effects (Ravera 1991; Salánki et al. 2003). Due to this sensitivity, pulmonate snails are considered as excellent bioindicators of heavy metal contamination (Oehlmann & Schulte-Oehlmann 2003).

Animal embryos in particular had been recognized as a valuable and cost-effective tool for monitoring water quality because this life stage generally responds quickly to lower toxicant concentrations (Cheung & Lam 1998; Leung et al. 2007; Jezierska et al. 2009). Developmental patterns and hatching success can also serve as good proxies for estimating the ecological significance of sublethal levels of heavy metals and other pollutants (Kosalwat & Knight 1987). Among freshwater animals, zebrafish, *Brachydanio rerio* (Dave & Xu 1991) and common carp, *Cyprinus carpio* (Jezierska & Slominska 1997) are widely used for vertebrate embryo toxicity bioassays while *Lymnaea stagnalis* (Gomot 1998) and *L. luteola* (Khargarot & Das 2010a) are well established as the invertebrate counterpart. However, because of the species-specific responses to heavy metals and other pollutants, it is still very necessary to screen as many potential local animal alternatives specially on areas where these model animals are not available.

One of the best candidate bioindicators is *Radix quadrasi* von Möellendorf 1898, a native and common freshwater pulmonate snail found in ponds, rivers, lakes, and rice paddies in the Philippines (de Lara & Enriquez 1981). They serve as natural food source for fish and ducks, and known to be an intermediate host of the liver fluke, *Fasciola gigantica*. As a model animal for embryo toxicity studies, it has several advantages. This snail has a known life span (155-210 days), and can be reared easily under laboratory conditions with low maintenance. Adult snails can be induced to produce numerous transparent eggs (ranging from 20-60 eggs/ gelatinous mass) where various developmental stages can be observed clearly under the microscope. Its incubation period is short (7-10 days) with a high hatching success (98-100%) (de Chavez & de Lara 2003). Furthermore, this gastropod is a close phylogenetic relative of other lymnaeid bioindicators such as *L. stagnalis* and *L. (Radix) luteola* (Remigio 2002; Correa

et al. 2010) whose responses to various toxicants are already well documented. In this paper, we determined the toxic effects of heavy metals such as As^{3+} , Al^{3+} , Ni^{2+} , and Cr^{6+} on *R. quadrasi* embryos. Specifically we examined the acute and sublethal toxicity of each heavy metal and evaluated its sensitivity against the standards and detected field levels in polluted Philippine freshwater ecosystems.

METHODS

Test Organism

Adult *R. quadrasi* with shell size 13-16 mm were collected from the University of the Philippines Los Baños (UPLB), Limnological Research Station and were acclimatized and reared at the Animal Biology Research Laboratory, Institute of Biological Sciences, UPLB following the method of de Lara and Enriquez (1981) and de Chavez and de Lara (2003). Five to six snails were placed in each plastic basin (33 cm x 25 cm x 11 cm) filled with 3 L aged tap water at room temperature (25°C) in a 12h light:12h dark cycle to minimize change in the snails' circadian rhythm that might affect mating and egg-laying behavior (Souza et al. 1988). The basins were lined with strips of filter paper prepared two days prior to the introduction of snails to permit the growth of microalgae. The snails were fed with fresh lettuce leaves (*Lactuca sativa*) *ad libitum*. The snails' excreta and unconsumed food were removed regularly. Water in the basin was changed twice a week. Aeration was constantly supplied to prevent the medium from fouling. Laid egg masses were randomly placed in Petri dishes where embryos were allowed to reach early trochophore stage (2 days after oviposition) (Table 1).

Physico-Chemical Parameters of Solvents

The physico-chemical characteristics of deionized and aged tap water solvents were determined before toxicity tests. Aged tap water is water allowed to stand for at least 72 hrs at room temperature (25°C) for the complete degradation of free chlorine (Teefy & Singer 1990). Water temperature and pH were measured using a digital thermometer and Kay-May Combination pH electrodeTM, respectively. Hardness and alkalinity were analyzed using EDTA titrimetric methods. The deionized water had a pH of 7.20 ± 0.01 and alkalinity at 1.21 ± 0.00 mg/L $CaCO_3$ at 26°C while the aged tap water had a pH of 7.81 ± 0.01 and alkalinity of 146.78 ± 1.46 mg/L $CaCO_3$ at 26°C. No other heavy metals were detected among the water samples tested.

Preparations of Stock and Treatment Solutions

Stock solutions were prepared by dissolving pure analytical grade 2.2073g $NiCl_2$ (>98% - Sigma) 4.1656g Na_2HAsO_4 • $7H_2O$ ($\geq 98.0\%$ - Sigma), 2.8289g $K_2Cr_2O_7$ ($\geq 99.5\%$

Table 1. Normal embryonic development and corresponding time of onset in *Radix quadrasi* at 25°C (de Lara & Enriquez 1981; de Chavez & de Lara 2003).

Embryonic stage	Time* (after oviposition)	Description
First polar body	5-10 mins	Small mass budding off from the fertilized embryo
First cleavage	1.5 - 2.5 hrs	Holoblastic spiral cleavage, embryo is small, opaque, circular, no motion
Gastrula	14- 16 hrs	Embryo is a dark cluster of cells, very slow in motion
Trochophore larva	22- 40 hrs	Embryo with bilobed vesicle in the former region of the animal pole, increases in size, ciliated, consistent rotating movement within egg capsule
Veliger larva	3-3.5 days	Marked body elongation, curvature of the head-foot region, protoconch starts to be visible, heart pulsation towards the end of the post veliger stage
Hippo stage (miniature snail)	4-5 days	Enlarged head-foot region distinctly separated from the visceral mass, formation of the tentacles above the dark pigmented eye, heartbeat and general body movement more observable, periodic contraction of visceral mass towards the head-foot region, embryo starts to creep on the inner membrane of the egg capsule

*duration could vary depending on temperature

- Sigma), and 12.3492g $Al_2(SO_4)_3 \cdot 18H_2O$ ($\geq 98.0\%$ - Sigma) in 1 L deionized water to make 1000 mg/L ionic solutions. Each solution was stored in new polyethylene plastic containers to prevent metal adsorption. The working treatment solution was prepared daily by serial dilution from the stock using aged tap water as the diluent.

Range Finding Test

Static renewal was conducted on *R. quadrasi* embryos for the range finding test (RFT). Egg masses were examined under a light microscope to determine pre-existing abnormality. Egg masses with 30 to 40 embryos were placed in individual plastic Petri dishes and were exposed to nominal concentrations (100, 10, 1, 0.1, 0.01, 0.001, and 0.0001 mg/L) of the heavy metals for 96 h to determine the possible working solutions that will be used in the definitive acute and sublethal toxicity tests. Aged tap water was used for the control group. Each treatment had five replicates with 30mL of freshly prepared solution.

Acute Toxicity Test

Similar procedure was used for the definitive acute toxicity test utilizing the estimated range from the RFT. Embryos were exposed to adjusted concentrations of As^{3+} , Al^{3+} , Cr^{6+} , and Ni^{2+} , that yielded partial kills. Each experimental set-up had five replicates (30-40 embryos) and was conducted three times. The viability of embryos was checked and recorded every 24 hours. Parameters used to determine dead embryos were coagulation, immobility, and loss of heartbeat. The lethal concentrations (LC_{50} -96h and LC_{10} -96h) for each heavy metal was calculated using probit analysis (Finney 1971).

Sublethal Toxicity Test

Embryos were exposed for 14 days (4-day extension from the maximum normal 10-day incubation period for *R. quadrasi*) using semi-static renewal toxicity test (Cheung & Lam 1998). Five concentrations for each heavy metal were used based on

the LC_{10} -96h. Each set-up had five replicates (30-40 embryos) and was conducted three times. Individual embryos which exhibited abnormalities and deformities were identified and counted. Sample abnormal embryos were photographed under a Nikon H-III Optihot-2 microscope. Hatchability and incubation period were also determined for the entire 14 day exposure period. Hatchability is the number of embryos that successfully emerged from egg capsule divided by the total number of eggs in a single egg mass whereas incubation period is the length of time from oviposition to the onset of hatching.

Statistical Analyses

All data were presented as mean \pm SD and were tested for normality and homogeneity of variance while percentages were arcsine-transformed prior to further analysis. One-way analysis of variance (ANOVA) with Tukey HSD post hoc was used to determine any significant differences in developmental abnormalities and incubation period among treatments. Repeated- measures ANOVA was used to determine significant difference in percent hatchability among days during the hatching period (10-12th day) including the 2-day extension. All statistical analyses were conducted using Statistical Package for Social Sciences (SPSS) for Windows (version 11.5.0, SPSS, Chicago, Illinois, USA).

RESULTS

Individual Metal Toxicity

The median lethal concentration (LC_{50} -96h) of the heavy metals (Table 2) on *Radix quadrasi* embryos showed that Cr^{6+} and Al^{3+} were the most toxic and the least toxic heavy metal, respectively. The toxicity trend observed was Cr^{6+} (0.0263 mg/L) > As^{3+} (1.0147 mg/L) > Ni^{2+} (1.5877 mg/L) > Al^{3+} (1.8787 mg/L).

Table 2. Median lethal concentration (LC₅₀) after 96 hours' exposure of *Radix quadrasi* embryos to arsenic (As³⁺), aluminum (Al³⁺), chromium (Cr⁶⁺) and nickel (Ni²⁺).

Heavy metal	LC ₅₀ -96h* (mg/L)	Mean confidence interval (mg/L)	
		Lower Limit	Upper Limit
As ³⁺	1.0147	0.1183	11.7250
Al ³⁺	1.8787	0.5333	10.2157
Cr ⁶⁺	0.0263	0.0023	0.8113
Ni ²⁺	1.5877	0.2867	27.3897

*overall mean of 15 replications (3 trials with 5 replicates each)

Sublethal Toxicity Test

Developmental Abnormalities

All control snails showed normal development. However, embryos exposed to sublethal concentrations of As³⁺, Al³⁺, Cr⁶⁺ and Ni²⁺ exhibited growth retardation, edema and thinning of the shell (Figure 1). Growth retardation was the most prevalent among embryos exposed to As³⁺, Al³⁺ and Ni²⁺ 14 days after (Figure 2). One-way analysis of variance (ANOVA) revealed significant difference ($p \leq 0.001$ for As³⁺, Al³⁺ and Ni²⁺ and $p \leq 0.05$ for Cr⁶⁺) in percentage of stunted embryos as compared to the control group. In Ni²⁺-treated embryos, there was a significant ($p \leq 0.05$) decrease in percent growth retardation between 0.0882

mg/L and 0.0887 mg/L (Figure 2). However at higher concentration (0.0897 mg/L), an increase in percentage of stunted embryos was again observed. In contrast to those exposed to As³⁺, Al³⁺ and Ni²⁺, edema was most observed among Cr⁶⁺-treated embryos. Thinning of the shell was the least frequent abnormality among *R. quadrasi* embryos exposed to sublethal concentrations of As³⁺, Al³⁺ and Ni²⁺.

Hatchability and Incubation Period

Embryos exposed to heavy metals had lowered mean hatchability and prolonged incubation period after the 14-day exposure (Table 3). The result suggests possible dose-response dependence. One-way repeated measures ANOVA showed significant difference in percentage hatchability [As³⁺ (Wilks' Lambda=0.12, $F=48.07$, $p<0.0005$), Al³⁺ (Wilks' Lambda=0.12, $F=49.44$, $p<0.0005$), Cr⁶⁺ (Wilks' Lambda=0.12, $F=48.07$, $p<0.0005$) and Ni²⁺ (Wilks' Lambda=0.19, $F=28.69$, $p<0.0005$)] among days.

Comparison with Accepted Standards and Detected Field Levels

The present data on the concentrations of the heavy metals were comparable to the guideline limit set for drinking water by the World Health Organization (WHO 1998, 2003a,b, 2005, 2011) and acceptable values to the freshwater systems by the United States Environmental Protection Agency (US EPA 2009) (Table 4). The lowest observed effective

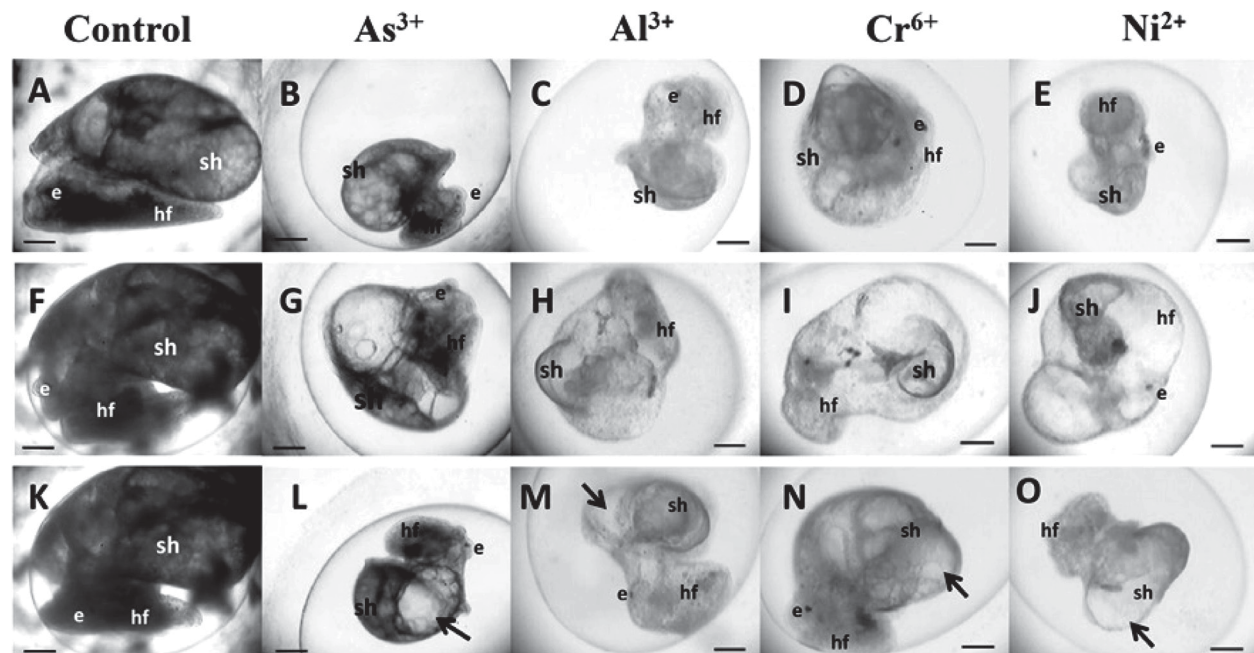


Figure 1. Photomicrographs of 10-day old *Radix quadrasi* embryos subjected to different heavy metal concentrations. Control embryos showed normal development (A,F,K); experimental embryos exposed to 0.0167 mg/L As³⁺, 0.045 mg/L Al³⁺, 0.0070 mg/L Cr⁶⁺, and 0.0897 mg/L Ni²⁺ showed growth retardation and shape deformity (B-E); edema (G-J) and thinning of shell (arrow) (L-O). e-eyes; hf- head-foot region; sh- shell. All scale bar = 5 μ m.

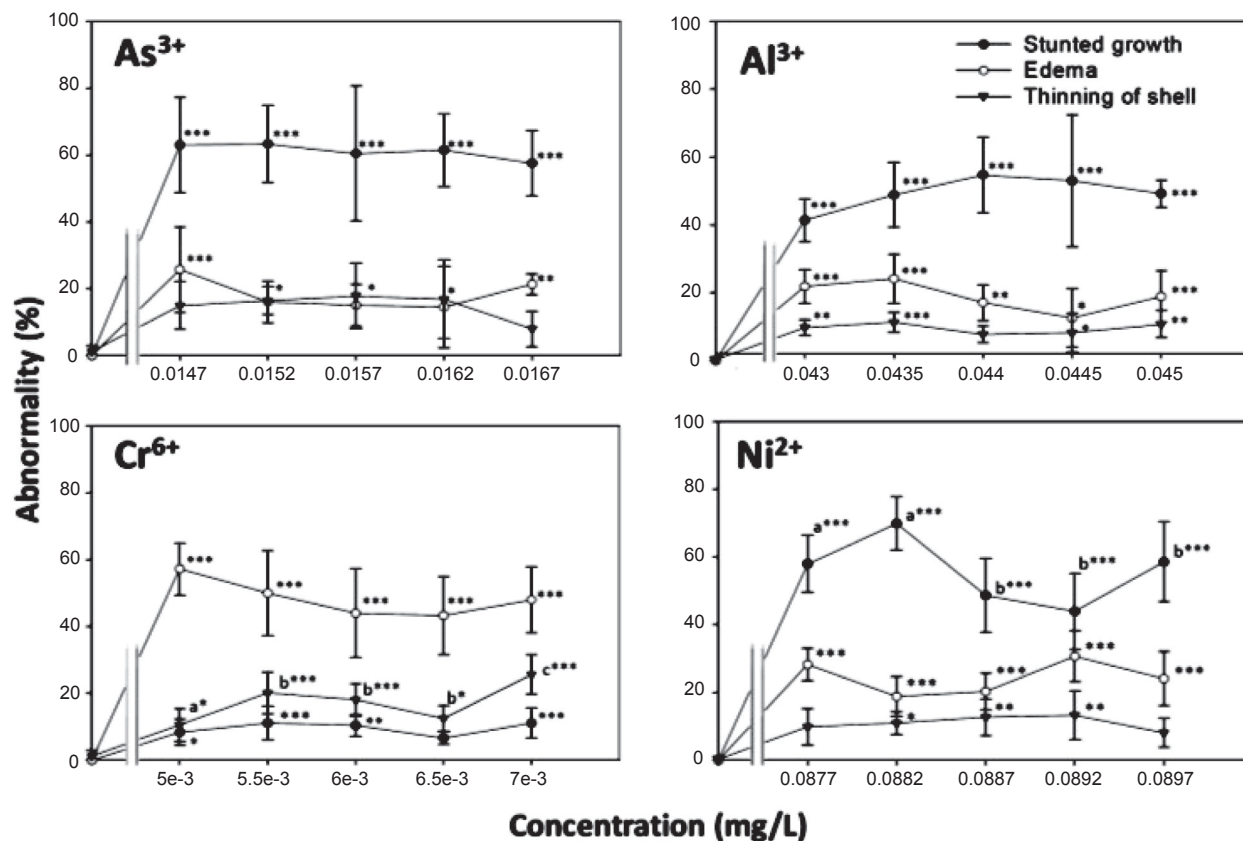


Figure 2. Frequency (mean \pm SD) of developmental abnormalities induced by As^{3+} , Al^{3+} , Cr^{6+} and Ni^{2+} on *Radix quadrasi* embryos after 14 days exposure. Different letters indicate significant differences between consecutive data points. Asterisk indicates significant difference from the control - * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

concentration (LOEC) of all heavy metals was also lower than the criteria continuous concentration (CCC). The LC_{50} -96h and LOEC for abnormality for Cr^{6+} were lower by 47.4% and 90%, respectively to the value set by WHO (200b). The LOEC of Al^{3+} was higher by 78.5% than the acceptable limit (0.200 mg/L) set by the Department of Environment and Natural Resources (DENR) of the Philippines (1994).

Discussion

Our study showed the acute sensitivity of *R. quadrasi* embryos to the four heavy metals after 96h. Heavy metal generally acts as a mitotic poison and as morphogenetic inhibitor interrupting cell metabolism, and water and ion balance (Gomot 1998). The change in the permeability of the gelatinous membrane and egg capsule surrounding the *R. quadrasi* embryo could also intensify metal ion access (de Chavez & de Lara 2003). Metal ions possibly diffused into the egg capsule fluid together with the nutritive materials and consequently were pinocytotically ingested by the embryos (Hess 1971).

At short term exposure, chromium (Cr^{6+}) was the most toxic heavy metal to *R. quadrasi* embryos. Cr^{6+} could have easily entered the egg capsule and pass through the snails' cell

membrane. It could have further reduced into Cr^{5+} and Cr^{3+} which directly targets the DNA bases and phosphodiester backbone causing mutagenesis and death (Marchese et al. 2008). These findings were similar to those of Trumble & Jensen (2004) which reported that Cr^{6+} was more toxic at the earlier stage of development in the insect *Megaselia scalaris*.

Arsenic (As^{3+}) could have reacted with mitochondrial enzymes of *R. quadrasi* embryos. Ansaldo et al. (2009) reported that As^{3+} could bind with -SH and -OH groups in the mitochondrial enzymes resulting in blocked synthesis and phosphorylation of proteins involved in several metabolic processes. As^{3+} could also inhibit ATP synthesis by acting on the enzymes involved in the citric acid cycle and phosphate transport system. This could then inactivate the pyruvate dehydrogenase and increase pyruvate in the hemolymph leading to gradual deterioration of the cell (Liao et al. 2004). As^{3+} was also reported to induce genetic disorders by replacing phosphorus in the DNA forming arsenodiester bond instead of phosphodiester bond thus inhibit DNA repair mechanism (Canivet et al. 2001; Mandal & Suzuki 2002). Canivet et al. (2001) observed As^{3+} as more toxic than Cr^{6+} in snail *Physa fontanalis* and in larvae of *Heptagenia sulphurea* and *Hydropsiche pelucidula*.

Table 3. Mean hatching and incubation period of *Radix quadrasi* embryos exposed to different heavy metal concentrations from day 10 to 14 ($n=5$).

Heavy Metals	Concentration (mg/L)	Hatching (%) at					Incubation Period (day)
		10-day	11-day	12-day	13-day	14-day	
As ³⁺	0.0147	0.57 ± 1.28	5.25 ± 1.94	11.22 ± 3.84	17.76 ± 5.76	28.63 ± 8.62	10.80 ± 0.45
	0.0152	0.00 ± 0.00	2.4 ± 2.28	8.46 ± 4.27	16.25 ± 8.36	23.82 ± 9.08	11.40 ± 0.55
	0.0157	0.00 ± 0.00	3.36 ± 1.92	8.36 ± 2.88	14.25 ± 3.96	20.57 ± 4.99	11.40 ± 0.55
	0.0162	0.00 ± 0.00	1.00 ± 2.24	6.10 ± 5.06	12.65 ± 6.00	20.55 ± 6.27	11.80 ± 0.45
	0.0167	0.00 ± 0.00	0.00 ± 0.00	1.83 ± 2.53	8.67 ± 5.37	19.70 ± 12.49	12.60 ± 0.55
Control	0.0000	23.00 ± 10.56	32.6 ± 11.01	35.80 ± 23.51	35.80 ± 23.5	35.80 ± 23.51	10.00 ± 0.00
Al ³⁺	0.0430	5.02 ± 5.47	16.90 ± 11.36	30.93 ± 12.23	42.58 ± 14.39	56.28 ± 18.37	10.20 ± 0.45
	0.0435	4.39 ± 4.79	12.14 ± 9.25	22.62 ± 10.09	32.51 ± 10.72	45.42 ± 11.47	10.20 ± 0.45
	0.0440	3.90 ± 2.96	11.04 ± 6.62	17.90 ± 7.18	27.57 ± 7.74	36.46 ± 12.29	10.40 ± 0.55
	0.0445	2.17 ± 1.46	7.17 ± 3.01	14.91 ± 5.22	22.70 ± 8.26	29.87 ± 13.70	10.40 ± 0.55
	0.0450	2.50 ± 2.29	7.52 ± 3.87	13.80 ± 5.39	20.27 ± 5.25	26.18 ± 7.14	10.40 ± 0.55
Control	0.0000	53.69 ± 7.55	78.39 ± 43.84	78.39 ± 43.84	78.39 ± 43.84	78.39 ± 43.84	10.00 ± 0.00
Cr ⁶⁺	0.0050	0.00 ± 0.00	0.34 ± 0.77	3.55 ± 1.07	6.58 ± 1.20	10.38 ± 4.69	11.80 ± 0.45
	0.0055	0.00 ± 0.00	0.74 ± 1.66	2.82 ± 1.72	4.90 ± 3.36	4.90 ± 3.36	11.33 ± 0.58
	0.0060	0.00 ± 0.00	1.57 ± 2.16	4.93 ± 5.29	4.93 ± 5.29	4.93 ± 5.29	11.75 ± 0.50
	0.0065	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	no hatching
	0.0070	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	no hatching
Control	0.0000	53.08 ± 6.41	89.50 ± 6.94	99.49 ± 1.02	99.49 ± 1.02	99.49 ± 1.02	10.00 ± 0.00
Ni ²⁺	0.0880	0.00 ± 0.00	6.86 ± 6.50	20.78 ± 7.94	33.16 ± 10.97	46.77 ± 14.53	11.40 ± 0.55
	0.0882	0.00 ± 0.00	0.00 ± 0.00	5.56 ± 3.90	16.44 ± 6.32	33.63 ± 3.71	11.80 ± 0.45
	0.0887	0.00 ± 0.00	2.04 ± 2.25	7.38 ± 0.92	12.62 ± 1.27	22.94 ± 5.27	11.40 ± 0.55
	0.0892	0.67 ± 1.49	3.34 ± 4.11	9.19 ± 5.87	14.63 ± 6.10	22.59 ± 6.23	11.20 ± 0.84
	0.0897	0.00 ± 0.00	0.94 ± 1.35	3.97 ± 1.60	7.80 ± 2.04	10.83 ± 3.69	11.60 ± 0.55
Control	0.0000	53.69 ± 7.55	78.39 ± 43.84	78.39 ± 43.84	78.39 ± 43.84	78.39 ± 43.84	10.00 ± 0.00

Table 4. Comparison of acceptable heavy metal concentration in drinking and freshwater with the present data on *Radix quadrasi* embryos.

Heavy Metal	WHO (mg/L)	United States EPA (2009)			Detected Levels in Philippine freshwater (mg/L)	Data from the present study	
		CMC ^a (mg/L)	CCC ^b (mg/L)	CMC ^(DENR 1994) (mg/L)		LC ₅₀ -96h (mg/L)	LOEC ^c (mg/L)
As ³⁺	0.010 ^(WHO 2011)	0.340	0.150	0.010	0.0017-0.0070 ^(Chavez et al. 2006)	1.0147	0.0147
Al ³⁺	0.200 ^(WHO 2003a)	0.750	0.087	0.200	0.0050-8.7100 ^(David 2003)	1.8787	0.0430
Cr ⁶⁺	0.050 ^(WHO 2003b)	0.016	0.011	0.050	13.200-27.1000 ^(Hallare et al 2005)	0.0263	0.0050
Ni ²⁺	0.070 ^(WHO 2005)	0.470	0.052	NA	4.7000-18.7000 ^(NEPC 1982)	1.5877	0.0088

^aCriteria Maximum Concentration

^bCriteria Continuous Concentration

^cLowest observed effective concentration which induced abnormality among embryos

Nickel (Ni^{2+}) showed intermediate toxicity to *R. quadrasi* embryos after the short term exposure. Several studies reported that Ni^{2+} induced toxicity by inhibiting Na^+ - Ca^{2+} exchange via ionic mimicry. Ni^{2+} can compete with Mg^{2+} and Ca^{2+} in the cell membrane resulting in longer cell repolarization which can lead to several functional disorders (O'Neill et al. 1988; Rainbow & Dallinger 1993; Bridges & Zalups 2005; Pane et al. 2006). Nickel-mediated oxidative damage to DNA and proteins could also cause inhibition of cellular antioxidants (Eisler 1998). Comparing life stages, the embryos of *L. stagnalis* were more sensitive to Ni^{2+} than the adults (Ravera 1991).

Aluminum (Al^{3+}) was the least toxic to *R. quadrasi* embryos. This could be due to the limited ions going into the body of the embryos during first three days of exposure when the snails' functional digestive system was yet to be developed. Al^{3+} enters the body mainly via oral ingestion. This metal could then accumulate in the gut and be stored inside the digestive cells as insoluble Al^{3+} complexes which cannot be excreted in the haemolymph and eventually destroying the digestive functions (Guibad & Gauthier 2003). Al^{3+} was also demonstrated to form polyhydroxy colloid which can adhere to mucus and interfere with osmoregulatory organs and lungs in the developing *L. stagnalis* (Dobranskyte et al. 2006).

In the sublethal test, the common teratogenic abnormalities exhibited by the embryos were edema, thinning of the shell and stunted growth. However, it is still unclear which among these developmental defects is more detrimental and will result in more mortality. Extended examination beyond the 14-day period of these abnormal embryos could address this issue. It seemed, however, that the observed abnormalities were not heavy-metal specific since *R. quadrasi* embryos treated with Zn^{2+} and Pb^{2+} also exhibited growth retardation, edema, and very thin shells (de Chavez and de Lara 2003). Cheung and Lam (1998) also reported stunted growth as most conspicuous at higher Cd^{2+} concentration (0.50 mg/L) on *P. acuta*.

The observed fluctuations in the developmental abnormalities particularly among Ni-treated embryos could be due to the activation of the snail's internal defense mechanism such as metallothioneins which could suppress Ni^{2+} from eliciting toxic effects (Ringwood & Brouwer 1993; Mason & Jenkins 1995). However, at the highest concentration (0.0897 mg/L Ni^{2+}), there was increased frequency of growth retardation among embryos suggesting interference on this mechanism. Similar observations were also reported on the freshwater snail *L. luteola* when exposed to Cd^{2+} (32 $\mu\text{g/L}$) (Khangarot & Das 2010b) and *Helix aspersa* (Coourdassier et al. 2000) subjected to increased Cr^{6+} .

Edema was the most common abnormality among Cr^{6+} -treated embryos. This could be due to the ability of Cr^{6+} to easily pass through the plasma membrane and be oxidized into Cr^{3+} in the cytosol as compared to other heavy metals

(Marchese et al. 2008). In this form, it cannot be transported back across cell membrane but will form complexes with nucleotides, proteins and organic compounds. The complexes will create an increased solute concentration in the cytosol which will form hypotonic condition by increasing the rate of water entry into the embryo resulting in bloating. In addition, Cr^{6+} and other metal ions and could also decrease the activity of Na^+ K^+ -ATPase causing ion imbalance contributing to impaired cell membrane function (Lushchak et al. 2008). In contrast, chromium at pH 6.3 was restricted to the egg shell of *C. carpio* embryos since insignificant levels were detected in the newly hatched larvae (Stouthart et al. 1995).

The shell thinning observed could be due to deficiency or unavailability of Ca^{2+} . Cr^{6+} is known to reduce the activity of Ca^{2+} -ATPase, an enzyme responsible for active calcium transport (Viyajavel et al. 2007). Ni^{2+} and Al^{3+} could also function as competitive inhibitors of Ca^{2+} (Zafar & Weaver 1999; Pane et al. 2006). Bridges and Zalups (2005) reported that these metals interfere with membrane transporters involving Ca^{2+} uptake through calcium channels. All heavy metal ions used could have competed with Ca^{2+} in this process and decrease CaCO_3 deposits in the shell (Grosell & Brix 2009).

The hatching process, stimulated by joint interactions of chemical, osmotic and mechanical mechanisms (de Lara 1991; Khangarot & Das 2010b), was inhibited by the heavy metals. The toxicants could have reduced the metabolic cost for growth and development leading to insufficient energy to perform the mechanical part of hatching such as rasping of the egg capsule by the head-foot region. De Chavez and de Lara (2003) found that hatchability of *R. quadrasi* was significantly reduced to 11.11% and 3.92% when exposed to Zn^{2+} (0.0001 mg/L) and Pb^{2+} (0.001 mg/L), respectively, while incubation period was lengthened to 2-5 days. Moreover, nickel along with copper was the strongest hatching-inhibitor among the metal-treated *B. danio* (Dave & Xu 1991) while delayed hatching was observed in *Salvelinus fontinalis* embryos upon exposure to aluminum at low pH (Cleveland et al. 1986).

The LOEC was generally comparable to the accepted values for public safety and reflective of the ecologically realistic levels detected in some polluted Philippine freshwater systems. Based on these results, *R. quadrasi* embryo can be classified as a sensitive general bioindicator (van Straalen 1998) capable of exhibiting symptoms of stress and damage such as mortality and developmental abnormality upon exposure to trace levels of heavy metals. This could have different implications. Because of its high sensitivity, it can provide wider environmental protection covering other less sensitive organisms, including humans and other vertebrates. Moreover, since *R. quadrasi* is a natural food of other vertebrates such as fish and ducks, there is always the possibility of bioaccumulation, even though starting at residual concentrations, which can still be detrimental as

heavy metals are stable and can be biomagnified at higher trophic levels (Goodyear & McNeill 1999). However, one should also be careful in adapting this high resolution bioindicator because it can potentially cause too frequent and rapid environmental alarm thereby compromising its long term warning function. One way of negotiating the differences between field data and laboratory experiments is the use of internal threshold concentrations such as lethal body concentration (LBC) (Van Wensem et al. 1994) which can estimate species-specific heavy metal residues between field and laboratory model organisms.

RECOMMENDATIONS

For future researches, it is recommended to conduct acute and sublethal toxicity bioassays focusing on other life stages of *R. quadrasi* such as juveniles and adults. Moreover, additional parameters like rate of heartbeat, eye, and tentacle formation could be adapted to provide more in depth information on the effects of toxicants. Use of histopathological techniques and molecular biomarkers (e.g. metallothioneins) should also be explored in order to understand the specific targets of the heavy metals at the cellular level. We do acknowledge that it is too early to recommend specific and safe exposure levels for each of the tested heavy metals on the sole basis of this preliminary embryotoxicity bioassay using *R. quadrasi*. However, this kind of study can jumpstart future related projects using invertebrate biomonitors geared towards reassessment of the acceptable levels of heavy metals in Philippine freshwater systems for better water quality monitoring with wider range of environmental protection.

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