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Embryotoxicity of Copper and Zinc in Tropical Sea Urchin *Tripneustes gratilla*

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ABSTRACT

The study determined the individual toxicity of copper (Cu) and zinc (Zn) in sea urchin *Tripneustes gratilla*. Bioassay using inhibitions on fertilization, early cleavage, mid cleavage, late cleavage and blastulation as endpoints involved exposure of viable gametes to Cu and Zn for 0.5, 3, 6, 9 and 12 h, respectively. Inhibitions increased significantly with concentration of Cu and Zn. Probit analysis estimated EC_{50} values for Cu and Zn, respectively, at 32 and 67 $\mu g \cdot L^{-1}$ on fertilization; 31 and 93 $\mu g \cdot L^{-1}$ on early cleavage; 43 and 61 $\mu g \cdot L^{-1}$ on mid cleavage; 42 and 42 $\mu g \cdot L^{-1}$ on late cleavage; and 20 and 44 $\mu g \cdot L^{-1}$ on blastulation. Results showed that toxicity of Cu is significantly higher ($p<0.05$) than that of Zn in all developmental stages, except in late cleavage. Also, the inhibitions elicited by Cu showed sensitivity to life stages. This study provided evidence on heavy metal species-sensitive, concentration-dependent and stage-specific inhibitions on embryonic development in *T. gratilla* to Cu and Zn.

Keywords: Embryotoxicity, sea urchin development, individual toxicity, heavy metals

INTRODUCTION

Waste disposal from mines and industries discharges complex mixtures of pollutants to coastal areas. These anthropogenic activities expose aquatic wildlife to various heavy metals such as copper and zinc (US EPA 2007), which at elevated levels often subject aquatic organisms to heavy metal poisoning (Eisler 1998). Sea urchins dwell in marine environment, and they respond readily to heavy metal pollution, making them an ideal bioindicator of ecosystem health (Kobayashi and Okamura 2004).

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Known to act as teratogen, heavy metals cause developmental delay, malformations and mortalities among exposed aquatic organisms (Eisler 1998). Studies have found that different heavy metals cause developmental anomalies among sea urchins (Kobayashi and Okamura 2004), and elevated concentrations of Cu and Zn inhibit the development of echinoid species (Phillips and others 2003, Kobayashi and Okamura 2004, Kobayashi and Okamura 2005).

Although there have been several studies on the effects of toxic heavy metals on marine organisms (see for example King and Riddle 2001, Phillips and others 2003, Kobayashi and Okamura 2005), further research is needed to have a better understanding of the embryotoxic effects of heavy metals. Moreover, endpoints of sea urchin bioassay are limited to spermiotoxicity, inhibitions of fertilization, and malformations. Few studies have been undertaken to investigate the inhibitory effect of Cu and Zn on early stages such as cleavage and blastulation, which are critical stages in sea urchin development.

To help address these research gaps, the present study was undertaken. This study may be regarded as an initial attempt to evaluate the inhibitory effects of Cu and Zn on the early life stages of the tropical sea urchin, *Tripneustes gratilla*, in the Philippines. Using bioassay testing, this study aimed to: (a) determine the percentage of inhibitions on fertilization, early cleavage, mid cleavage, late cleavage, and blastulation; and (b) compare the inhibitions across heavy metal species, concentration, and developmental stages.

MATERIALS AND METHODS

Preparation of Test Solutions

Five nominal concentrations of Cu and Zn (0, 25, 50, 100 and 150 $\mu g \cdot L^{-1}$ each) were used to examine the toxicity of heavy metal on sea urchin. The test solutions were prepared by adding copper sulfate and zinc sulfate into filtered natural seawater. The temperature (28.10 ± 1.84°C), salinity (30.67 ± 0.58 $\mu g \cdot L^{-1}$), and pH (6.99 ± 0.6) of the test solutions were maintained.

Collection of Sea Urchin Gametes

Forty-two adult sea urchins *T. gratilla*, 6.60 ± 0.47 cm in diameter, were collected from Marigodon, Lapu-lapu City. Each organism was isolated in a plastic container filled with seawater to ensure that none of the sea urchins would induce others to spawn. They were transported to the laboratory immediately after sampling.

Procedures for gamete collection were adapted from the US EPA (1995) protocols, with a few modifications. Each sea urchin was inverted over a 100 mL beaker fully filled with filtered natural seawater. The gonadal openings on the aboral side were immersed in the seawater. About 1 mL of 0.5 M KCl was injected through the tough leathery peristomial membrane into the perivisceral cavity of each sea urchin. This resulted in the contraction of the smooth muscles of the gonad and induced spawning of the specimen. Injections were repeated after 2-5 minutes to induce heavier spawning. The sex of the sea urchin was determined. *T. gratilla* males ejected cream-colored semen while females released yellow eggs. A drop of the gametes from each sea urchin was examined under the microscope to confirm its sex.

Each spawning sea urchin male was transferred into a petri dish in oral side up position and was allowed to shed into the dish. A drop of the dry sperm (semen) was examined under the microscope to observe the motility of the sperm. The sea urchin males with high sperm motility were used in the test to ensure sperm viability. The viable sperm cells were pooled into a 100 mL beaker, which was covered with parafilm to prevent exposure of semen to air that may reduce the viability of the sperm by altering the surrounding pH. Sperm stock was stored at 5°C.

Female sea urchins were left to shed eggs into the 100-mL beakers filled with filtered natural seawater. A small sample of the eggs from each female was examined under the microscope to determine the presence of mature eggs. Mature eggs were characterized as having a) small nucleus found near the periphery of the cell membrane and b) large amount of cytoplasm. Mature eggs were pooled into a 1 L beaker. The eggs were suspended in 600 mL filtered seawater, and allowed to settle for 15 minutes. About 500 mL of the overlying water was siphoned off and the volume was brought back to 600 mL with filtered natural seawater. The eggs were resuspended and allowed to settle for 15 minutes. After siphoning off the overlying 500 mL, the eggs were finally resuspended in 600 mL filtered natural seawater. Egg suspension was stored at 12°C.

The gametes were used in the toxicity assay after 2 h following the collection. Gametes were exposed to different treatments of Cu and Zn (25, 50, 100 and 150 $\mu g \cdot L^{-1}$ each). Same batch of gametes were exposed to 0 $\mu g \cdot L^{-1}$ Cu and $\mu g \cdot L^{-1}$ Zn, which serve as control.

Toxicity Assay

The exposure experiments were adapted from the protocol used by Kobayashi and Okamura (2004, 2005), with a few modifications. Inhibitions on fertilization, early cleavage, mid cleavage, late cleavage and blastulation were the endpoints. Exposure experiment for every endpoint was conducted separately in a plastic container with 10 mL of test solution. One drop of dry sperm stock and 1 mL of the egg suspension were added into the container. The gametes that were exposed to different treatments were of the same batch. Incubation temperature (28 ± 2 °C), salinity (30 ± 1 $\mu g \cdot L^{-1}$), and pH (7 ± 0.5) were maintained throughout the exposure experiment. Fertilization, early cleavage, mid cleavage, late cleavage, and blastulation were arrested by adding 1 mL of 10% formaldehyde after 0.5, 3, 6, 9 and 12 h exposure to test solutions, respectively. Exposure experiments were triplicated.

A drop of the treatment solution was mounted on a slide. About four mounts were prepared for each treatment. Each mount was observed under the compound microscope in a single field of vision at 100x magnification. One hundred eggs and/or embryos were randomly selected and their development stage, as described in Table 1, was identified. Inhibitions on fertilization, early cleavage, mid cleavage, late cleavage, and blastulation were determined.

Table 1. Distinguishing features of early developmental stages in sea urchin

Stages	Description
Unfertilized Egg	Mature eggs without fertilization cone or envelope
Fertilized Egg	Mature eggs with fertilization cone or envelope
Early Cleavage	2- and 4-cell stage embryos
Mid Cleavage	8- and 16-cell stage embryos
Late Cleavage	32- and 64-cell stage embryos
Blastulation	Embryos with a sphere of cells surrounding a cavity

Data Analyses

The toxicity responses were reported as percent inhibitions on fertilization (IF), early cleavage (IEC), mid cleavage (IMC), late cleavage (ILC), and blastulation (IB) using the following formulas:

$$IF = \frac{U}{N}\, x\, 100 \qquad \text{Eq. 1}$$

$$IEC = \frac{U + F}{N} \; x \; 100 \qquad \text{Eq. 2}$$

$$IMC = \frac{U + F + EC}{N} \; x \; 100 \qquad \text{Eq. 3}$$

$$ILC = \frac{U + F + EC + MC}{N} \; x \; 100 \qquad \text{Eq. 4}$$

$$IB = \frac{U + F + EC + MC + LC}{N} \; x \; 100 \qquad \text{Eq. 5}$$

where *U* is the number of unfertilized eggs, *F* is the number of fertilized eggs, *EC* is the number 2- and 4-cell stage embryos, *MC* is the number 8- and 16-cell stage embryos, *LC* is the number of 32- and 64-cell stage embryos, and *N* is the total number of eggs and/or embryos evaluated.

The toxicity responses were fitted against the concentration through a probit model to estimate the concentration at which 50% inhibition is observed (EC_{50}). Kruskal - Wallis ANOVA was used to compare the toxicity responses among treatments. All statistical analyses were done using the IBM SPPSS Statistics Version 20 software, at 95% confidence interval.

RESULTS

The effects of varying concentrations of Cu and Zn on the different developmental stages of *T. gratilla* are shown in Figures 1 to 5. Both Cu and Zn elicited logarithmic concentration-dependent inhibitions on *T. gratilla* fertilization, early cleavage, mid cleavage, late cleavage and blastulation (Figures 1 to 5, respectively). Comparison between EC_{50} of Cu and Zn across different embryonic stages is shown in Figure 6.

Inhibitions on Fertilization (IF)

IF increased with increasing Cu and Zn concentration at 0.5 h exposure (Figure 1). All treatments of Cu and Zn elicited a significantly higher IF than in control (17 ± 7%). IF at 25 µg·L^{-1} Cu increased threefold from the control (62 ± 3%). Increasing the Cu concentration to 50 and 100 µg·L^{-1} elicited 70 ± 6% and 75 ± 3% IF, respectively, which were four times higher than in control. At 150 µg·L^{-1}, a fivefold increase in IF was observed (87 ± 3%). In zinc treatment, IF doubled at 25 µg·L^{-1} Zn (39 ± 3%). It increased threefold at 50 and 100 µg·L^{-1} Zn, eliciting 56 ± 3% and 61 ± 2% IF, respectively. It was significantly high at 150 µg·L^{-1} Zn (73 ± 5%), which was four

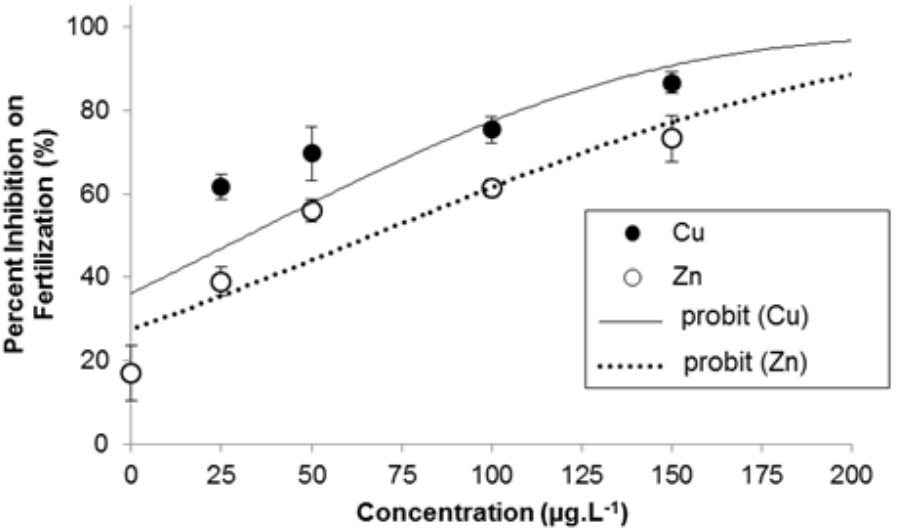

Figure 1. Inhibitions on fertilization to varying concentrations of Cu (black circles) and Zn (white circles).

times higher than in control. The EC_{50} of Cu (32 ± 11 µg·L^{-1}) was significantly lower than Zn (67 ± 3 µg·L^{-1}) (Figure 6), suggesting that Cu is twice as toxic as Zn in eliciting inhibitions on fertilization.

Inhibitions on Early Cleavage (IEC)

Concentration-dependent inhibitions on early cleavage of *T. gratilla* were also observed at 3 h exposure period to increasing Cu and Zn concentration (Figure 2). IEC in all Cu treatments were significantly higher than in control (15 ± 5%). At 25 µg·L^{-1} Cu, IEC increased threefold to 50 ± 6%. Increasing the concentration to 50 µg·L^{-1} Cu elicited 68 ± 7% IEC, which was four times higher than in control. IEC increased six times from the control at more elevated concentration (>90%). IEC at 25 and 50 µg·L^{-1} Zn showed no significant difference from the control. At higher Zn concentrations, IEC increased more than threefold, with values significantly higher than those in control. EC_{50} of Cu and Zn on IEC were 31 ± 3 µg·L^{-1} and 93 ± 43 µg·L^{-1}, respectively (Figure 6). There was a significant difference between the EC_{50} of Cu and Zn in eliciting inhibitions on early cleavage, indicating that Cu is three times more toxic than Zn.

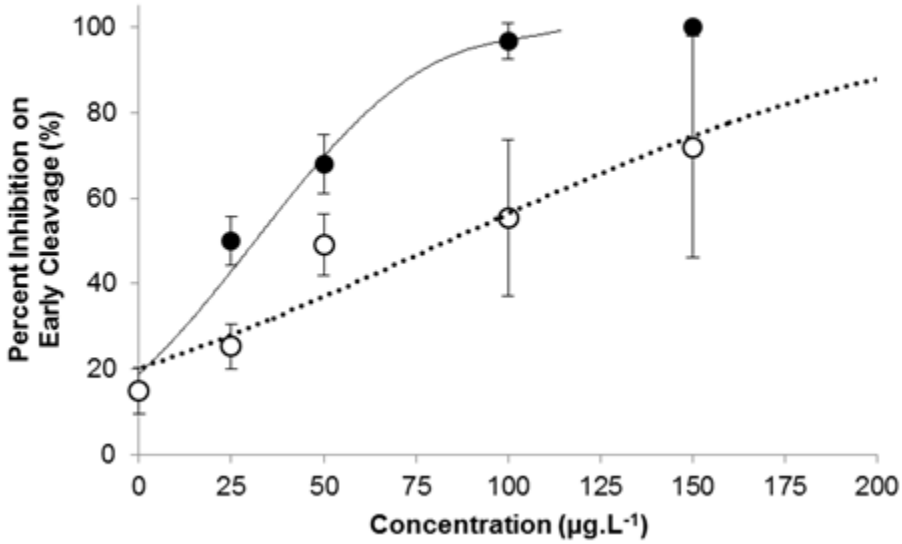

Figure 2. Inhibitions on early cleavage to varying concentrations of Cu (black circles) and Zn (white circles).

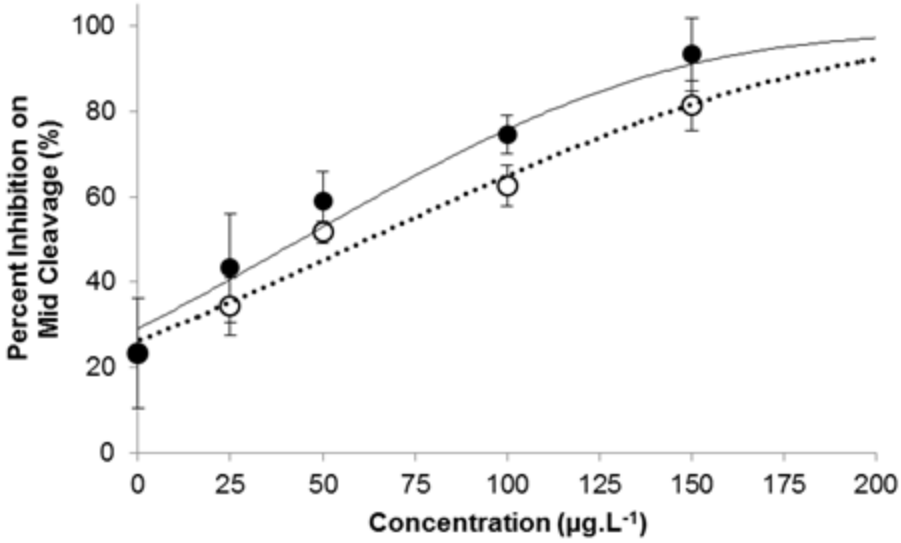

Figure 3. Inhibitions on mid cleavage to varying concentrations of Cu (black circles) and Zn (white circles).

Inhibitions on Mid Cleavage (IMC)

The inhibitions on mid cleavage were evaluated at 6 h exposure to varying concentrations of Cu and Zn (Figure 3). Similar to the previous observations, IMC increased with increasing concentration of Cu and Zn. IMC in both treatments did not vary significantly from the control (23 ± 13%) at 25 $\mu g \cdot L^{-1}$, but showed a significant difference from the control at elevated concentrations. In Cu treatment, IMC doubled at 50 $\mu g \cdot L^{-1}$ (59 ± 7%). Increasing the Cu concentration to 100 $\mu g \cdot L^{-1}$ elicited 75± 5%, which was threefold higher than in control. IMC increased four times at 150 $\mu g \cdot L^{-1}$ (93 ± 9%). There was a twofold increase in IMC at 50 and 100 $\mu g \cdot L^{-1}$ Zn (51 ± 3% and 63 ± 5%, respectively). At 150 $\mu g \cdot L^{-1}$ Zn, IMC had increased to 81 ± 6%, which was three times higher than in control. The EC_{50} of Cu (43 ± 11 $\mu g \cdot L^{-1}$) was significantly lower than Zn (61 ± 4 $\mu g \cdot L^{-1}$) (Figure 6), which suggests that Cu is more toxic than Zn in inhibiting mid cleavage.

Inhibitions on Late Cleavage (ILC)

Inhibitions on *T. gratilla* late cleavage were evaluated at 9 h exposure to Cu and Zn (Figure 4). Trends similar to those for the previous developmental stages were observed between ILC and concentration of Cu and Zn. All treatments showed a

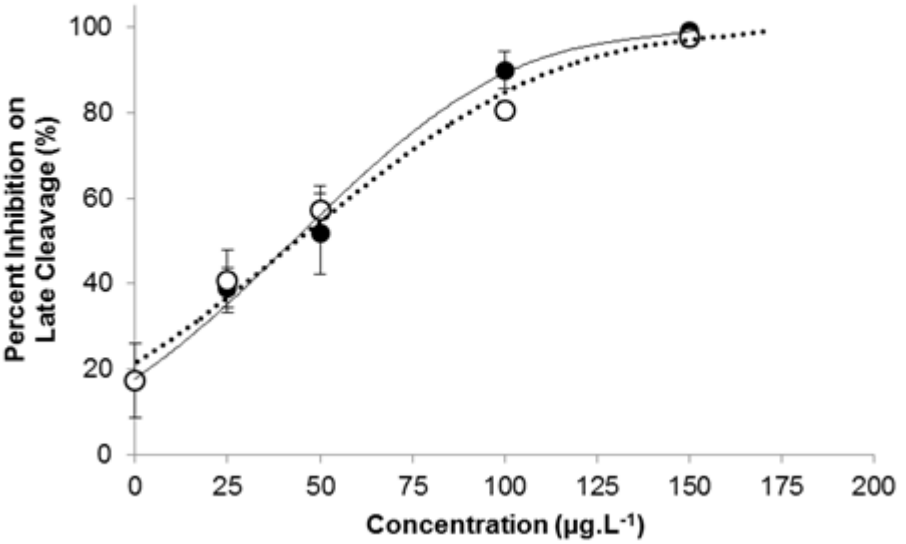

Figure 4. Inhibitions on late cleavage to varying concentrations of Cu (black circles) and Zn (white circles).

significant difference from the control (17 ± 7 %). ILC at 25 µg·L^{-1} Cu increased twofold from the control (39 ± 5%). Increasing the Cu concentration to 50 µg·L^{-1} caused 52 ± 10% ILC, which was three times higher than in control. At higher concentration, Cu elicited a fivefold increase in ILC (>90%). In zinc treatment, ILC doubled at 25 µg·L^{-1} Zn (40 ± 7%). It increased threefold at 50 µg·L^{-1} Zn, producing 57 ± 6% inhibitions. ILC at 100 µg·L^{-1} Cu quadrupled from the control (81 ± 2%). It was significantly high at 150 µg·L^{-1} Zn (98 ± 2%), which was five times higher than in control. Cu and Zn elicited EC_{50} at 42 ± 4% and 42 ± 9%, respectively (Figure 6). No significant difference was observed between EC_{50} of Cu and Zn, which indicate that Cu is as toxic as Zn. This is a different trend from that obtained in the previous observations.

Inhibitions on Blastulation (IB)

The percent inhibitions on blastulation were determined at 12 h exposure to varying concentrations of Cu and Zn (Figure 5). Similar trends of inhibitions were observed in the blastulation, which was found to increase with increases in the concentration of Cu and Zn. IB in all Cu treatments varied significantly from the control (17 ± 6%). At 25 µg·L^{-1} Cu, IB was quadrupled (70 ± 6%). At elevated Cu concentration, IB was five times higher compared to control (>82%). IB at low concentration of Zn showed

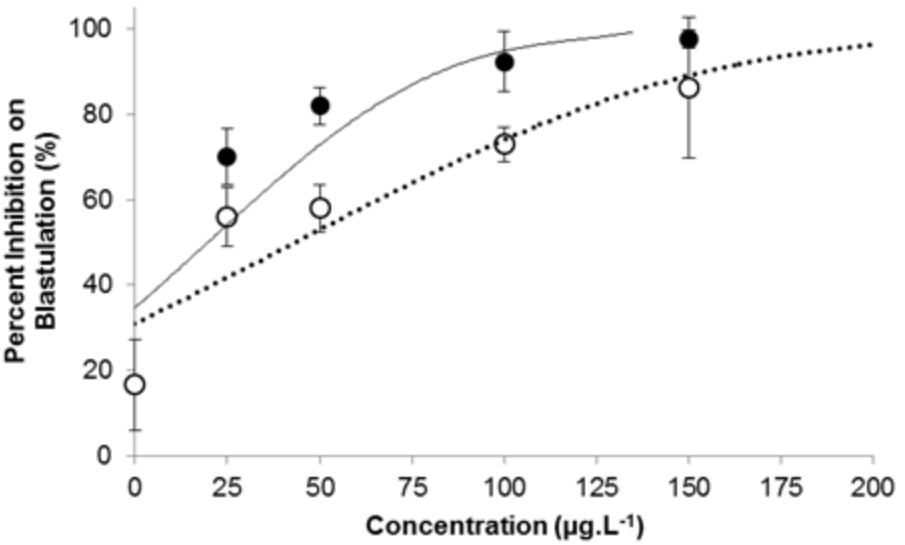

Figure 5. Inhibitions on blastulation to varying concentrations of Cu (black circles) and Zn (white circles).

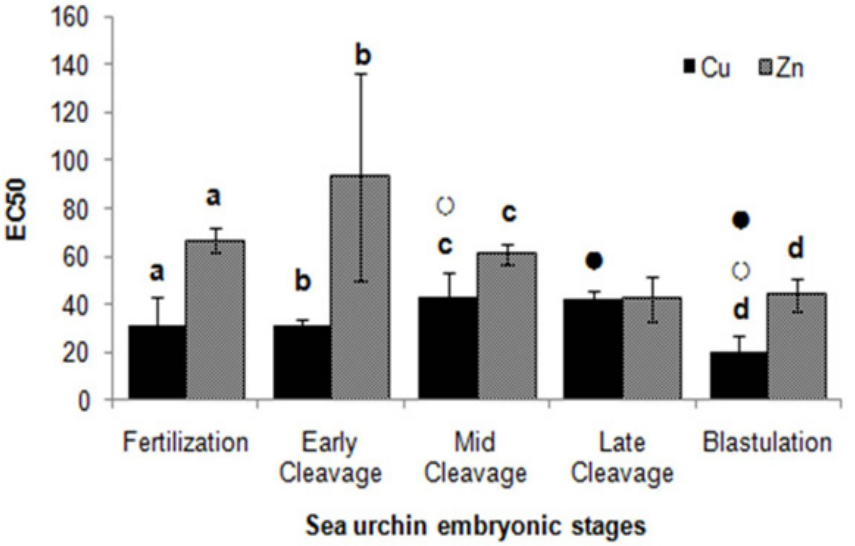

Figure 6. Comparison of EC_{50} values of Cu and Zn in eliciting inhibitions of different stages of embryonic development in sea urchin *T. gratilla*. Lower-case letters indicate significant difference between inhibitory effects of Cu and Zn for a particular embryonic stage. Black and white circles indicate significant difference between the inhibitions of two stages for a certain heavy metal.

no significant difference from control. Concentration at 100 $\mu g \cdot L^{-1}$ Zn elicited an IB of 73 ± 4%, which was four times higher than in control. Increasing the concentration to 150$\mu g \cdot L^{-1}$ Zn increased the IB to five times (86 ± 17%). The EC_{50} of Cu and Zn were 20 ± 7 $\mu g \cdot L^{-1}$ and 44 ± 6 $\mu g \cdot L^{-1}$, respectively (Figure 6). Based on this EC_{50} values, Cu appeared to be twice as toxic as Zn in inhibiting blastulation.

DISCUSSION

Industrial and agricultural wastes discharge heavy metals such as Cu and Zn that pollute coastal areas and endanger aquatic organisms, including the sea urchins. Elevated concentrations of these heavy metals may cause adverse effects on the growth, survival and reproduction of the echinoid species (Thongra-ar 1997, Kobayashi and Okamura 2004). Findings of this study showed that elevated concentrations of Cu and Zn lead to significantly higher inhibitions on fertilization, early cleavage, mid cleavage, late cleavage and blastulation in *T. gratilla*. The significant variation of percent inhibitions between control and treatments manifests toxicity of Cu and Zn in early developmental stages. Previous studies reported similar findings on embryotoxicity of heavy metal when elevated from their natural

concentrations in seawater (US EPA 1987, Nakamura and others 1989, King and Riddle 2001).

The observed inhibitions on fertilization can be attributed to the spermiotoxic effects of Cu and Zn. Motility and fertilizing capacity of *T. gratilla* spermatozoa are reduced by these toxicants (Thongra-ar 1997), hence lowering the fertilization success. Results also revealed inhibitions on cleavage and blastula stages, which are clear mitotoxic responses of Cu and Zn. Exposure to high levels retards the division of cells, thus delaying the formation of blastula (Kobayashi and Okamura 2004).

Copper may elicit inhibitions on the early life stages in sea urchin by (1) respiratory acidosis (Bielmyera and others 2005) or (2) disruption of ionic balances by alteration of ATPases (Li and others 1996) and carbonic anhydrase (Zimmer and others 2012). Zinc, on the other hand, possibly causes embryotoxic effects by: (1) inhibition of glucose-6-phosphate dehydrogenase that transforms carbohydrate via the pentose phosphate pathway (Durkina and Evtushenko 1991), (2) inhibition of the synthesis of ribosomal RNA (Pirrone and others 1970), (3) restriction of the development of endoderm as well as mesenchyme derivatives causing abnormalities to developing embryos (Timourian 1968), and (4) intervention with the action of cortical granule-derived protease that inhibit the formation of the fertilization membrane in sea urchin eggs (Nakamura and others 1989).

The results of this study provide evidence of concentration-dependent inhibitions caused by Cu and Zn on the embryonic development of sea urchin (Figures 1 to 5). Generally, inhibitions on developmental stages in sea urchin followed a logarithmic pattern when plotted against the concentration of Cu and Zn. Inhibitions increased exponentially at low concentration but slowed at elevated concentration.

Although the effects of other stressors (e.g. particulate materials) on the inhibitions could not be completely excluded, these were minimized in the experiment. In fact, samples of natural seawater were collected in a pristine area and were filtered to remove particulate materials. Hence, any variation between the nominal and actual would have been kept at minimum; the same would be true for the observed concentration-response relationships.

One limitation of this study is that inhibitions in the control group (>15%) were higher than the value (≤10%) ideal for toxicity testing. As such, it could be argued that sources of stress other than Cu and Zn could have contributed to the

concentration-response relationship observed. Also, levels of Cu and Zn were not determined in the test solutions, consequently casting some questions regarding the accuracy of the EC_{50} values. Corollary to this, some inhibitions in Cu treatments showed higher than 50% for all concentrations (e.g., Figures 1, 2 and 5). A possible explanation for this is that the EC_{50} might be overestimated due to lack of information on the inhibitions at concentrations lower than the tested levels.

It must be pointed out, however, that although range finding tests were not conducted before the definitive tests, the exposure experiments were designed to determine inhibitions at concentrations within the range of the EC_{50} values specified in the literature (see for example Kobayashi 1990, King and Riddle 2001, Phillips and others 2003). Hence, any differences in the threshold values between experiments with or without range finding test would be insignificant. There would be a negligible difference in EC_{50} values between definitive tests conducted with range finding test and definitive tests conducted without range finding test since the concentration used in the definitive tests were at concentrations within the range in the literature.

As observed, Cu was toxic within the range of 20 to 43 $\mu g \cdot L^{-1}$. EC_{50} of Cu. Comparatively, this is below (King and Riddle 2001, Phillips and others 2003), within (Heslinga 1976, Pagano and others 1986) and above (Kobayashi 1985, Ramachandran and others 1997) the observed threshold range reported in other studies. On the other hand, EC_{50} of Zn from the past studies is below (Kobayashi 1990), within (Phillips and others 2003) and above (Bay and others 1993, Thongra-ar 1997, King and Riddle 2001) the observed range of EC_{50} of Zn (42 - 93 $\mu g \cdot L^{-1}$). Findings showed the sensitivity of sea urchin bioassay to the heavy metal species (Figure 6).

Generally, EC_{50} of Cu was significantly lower than that of Zn in all developmental stages, except in late cleavage. This comparison of Cu and Zn toxicity tests on developmental stages in *T. gratilla* suggests that Cu is more toxic than Zn. The potential toxicity of Cu to *T. gratilla* was found to be 2–4 times greater than Zn. Previous studies also observed the same trend (Thongra-ar 1997, Kobayashi and Okamura 2004 and 2005).

Responses of embryonic development to toxicants are stage specific (Pagano and others 1986, Dinnel and others 1987, Bay and others 1993). This can be seen in the findings regarding the inhibitions caused by Cu in the different developmental

stages of *T. gratilla* (Figure 6). EC_{50} of Cu in blastulation was significantly higher compared to the threshold value in mid and late cleavage, suggesting that Cu is more toxic during blastulation than during the earlier developmental stages. Kobayashi (1980) reported that Cu is more disruptive in the later embryonic stages than in the earlier stages. In contrast to the findings for Cu, inhibitions elicited by Zn did not vary significantly across developmental stages, which indicate that the toxicity of Zn is not stage-dependent.

Accumulative trend in the inhibitions from early to late embryonic development was not observed in the present study. That is, it appears that the toxicity endpoints are independent of each other. The inhibitions in early development do not seem to influence the inhibitions in the latter stages. One possible explanation is that heavy metal exposure experiments were not carried out continuously. In continuous bioassay testing, it is expected that inhibitions from fertilization to blastula will show remarkable differences.

CONCLUSION

The study examined the inhibitory effect of Cu and Zn on fertilization, early cleavage, mid cleavage, late cleavage and blastulation of *T. gratilla*. The inhibitions exhibited logarithmic concentration dependence where it increases exponentially at low concentration, but more slowly at elevated concentrations of heavy metals. The findings confirmed the sensitivity of sea urchin bioassay to heavy metal pollution, with Cu eliciting greater toxicity than Zn in the early developmental stages of *T. gratilla*. Also, the study revealed the sensitivity of the assay to the developmental stages, although only Cu showed stage-specific inhibitions. Generally, the study provided a clear evidence of the dependence of heavy metal toxicity on heavy metal species, their concentration and the developmental stage they inhibit. The findings may contribute to the improvement of the bioassay, particularly the use of the sea urchin *T. gratilla* in the assessment of toxicity of harmful anthropogenic substances.

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