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Original Article

Development of a Toxicity Bioassay Using Fertilisation in the Green Mussel, *Perna viridis*, from Exposure to Copper and Cadmium

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Abstract. Previous studies have shown marine bivalves to be suitable bioindicators for heavy metal pollution in the marine environment. In this study, the potential of the Green Mussel, *Perna viridis* to be a bioindicator of copper and cadmium contamination in the marine environment was explored. The study aimed to develop a toxicity bioassay using *P. viridis*, and explored if a dose-response relationship between fertilisation in *P. viridis* and increasing concentrations of copper and cadmium could be established. The effect of copper and cadmium on embryo formation was also examined. Few fertilisation assays involve the use of the *P. viridis*, a tropical marine bivalve. Rather, a temperate mussel *Mytilus* sp., is more commonly used. As such, this study is relevant to the Southeast Asian region. Mussel gametes were subjected to increasing concentrations of cadmium and copper over a 24h test period. Following this, the number of unfertilised eggs was counted under a microscope. Data obtained was then arcsine transformed and subjected to one way Analysis of Variance (ANOVA) and Dunnett's test to determine if a significant dose-response curve could be established. Results indicated significant treatment effects and a degeneration of unfertilised eggs when gametes were subjected to cadmium and copper solutions.

Keywords: Ecotoxicology, *Perna viridis*, pollution, fertilisation bioassay.

Introduction

Pollution from heavy metals is becoming a severe threat to the marine environment in Southeast Asia as human populations expand. Release of industrial effluents into the environment is largely responsible for the pollution of coastal regions. As such, trace amounts of copper and cadmium are becoming increasingly prevalent in coastal regions. These heavy metals have the potential to become persistent metallic compounds and accumulate in marine organisms, threatening human health if they are consumed. Although trace metals are naturally found in the biosphere, elevated levels of copper and cadmium may be toxic to marine organisms (White and Rainbow, 1985; Viarengo, 1989). Increased exposure to these heavy metals may result in a physiological change to the organisms (Akberali and Trueman, 1985). Therefore, it is important to establish procedures for monitoring these pollutants that pose a threat to humans and the biota (Ringwood, 1992).

Many toxicity bioassays have been developed to monitor the presence of pollutants in the marine environment, employing oysters, mussels, sea urchins, sand dollars, fishes and mysids as test organisms (ASTM, 1995; Nipper *et al*., 1997; King and Riddle, 2001; Carr and Nipper, 2003). There has been extensive study of the biological effects of heavy metals on bivalves. Multiple studies have shown that marine bivalves may be used to indicate the level of environmental pollution in their habitat (Phillips, 1977; Bryan, 1979). Marine bivalves have shown to be able to accumulate high concentrations of trace metals. This ability resulted in the establishment of the Mussel Watch in the United States in the 1970s, and Mussel Watch served as a biomonitor for the marine environment (Farrington, 1983). Toxicity bioassays allow for trace amounts of metals to be detected, as well as their impact on a particular organism to be observed.

Although environmental toxicants can affect all life stages of marine invertebrates, it has been demonstrated that the earlier life stages of marine invertebrates are more responsive to toxicants than adults (His *et al*., 1999). Fertilsation inhibition assays measure the extent of reduction in fertilisation when gametes are exposed to pollutants (Gunthorpe *et al*., 1995). Fertilisation inhibition tests have been shown to be successful in determining the toxicity of heavy metals, petroleum, petroleum products, pesticides and complex effluents (Allen 1971; Hagstrom and Lonning, 1977; Dinnel *et al*,. 1982; Kobayashi, 1981; Cherr *et al*, 1987; Dinnel *et al*, 1989; Higashi *et al*, 1992). Exposure to increased levels of copper and cadmium has been shown to reduce viability of gametes in marine bivalves (Fitzpatrick *et al*., 2008).

Previous studies have focused largely on the biological impact of heavy metals on mussels of the *Mytilus* sp. while not many studies employed the use of tropical and subtropical species. The blue mussel *(Mytilus trossulus)* is the most commonly used species in toxicity assays, as it is a member of the most sensitive genus in the US EPA's database for saltwater copper quality criteria. However, it is mainly found in polar and temperate waters. Mussels in the genus *Perna* are found almost exclusively in the Southeast Asian region and *Perna viridis* may be considered as a subtropical counterpart of the *Mytilus* sp. (Siddall, 1980). The use of the green lipped mussel *(Perna viridis)* is more relevant to our bioassay as it is found in tropical waters in the Asian coastal regions. Both the *Perna viridis* bioassay and *Mytilus* sp. bioassay are advantageous as compared to bioassays employing other organisms as they are cost effective, do not require expensive equipment, are short term, taking hours or days rather than weeks for

completion, and are able to be carried out at all times of the year as the gametes are available at most times throughout the year (Johnson, 1988). Furthermore, the numbers of gametes that can be produced and used in mussel bioassays is large enough to allow good statistical analyses (Peters *et al*., 1997). *Perna viridis* has also been used extensively in the Indo-Pacific as a biomonitor and holds significant potential for further pollution monitoring (Nicholson, 1999).

For the purpose of this experiment, it is assumed that heavy metals have a definite impact on the fertilisation rate of *Perna viridis*. Specimens were subjected to increasing concentrations of copper and cadmium, following which the rate of success of fertilisation was ascertained. Consequently, statistical analysis was done to determine the concentrations of cadmium or copper in seawater which would cause a significant impact on the fertilisation success of *Perna viridis*. The main objectives of the study are to i) establish a dose-response relationship between fertilisation of the green-lipped mussel and copper and cadmium, and ii) observe the impact of copper and cadmium on embryo formation. Tests were carried out when mussels began spawning, and gametes collected.

Materials and Methods

Green Mussel Collection and Preparation

Gravid green mussels (*Perna viridis*) were collected from a local supplier at Tuas Jetty (1° 17' 12 N 103° 36' 52 E), Singapore, on the day of a full moon. In the laboratory, the mussels were cleaned thoroughly, which included removing fouling matter, barnacles and byssus threads from the shells. After rinsing, the mussels were put into an aerated holding tank with saltwater with salinity of 25 ± 1‰ and temperature of 21 ± 2°C. The saltwater was made by adding 1000 g of sea salt into 30 litres of water to obtain saltwater with an approximate salinity of 25 ± 1‰. Once gamete release was observed, the mussels were removed as soon as possible from the holding tank and placed individually into crystallising dishes for collection of gametes. Females were placed into crystallising dishes separately while males were placed together in a single crystallising dish. Once the mussels stopped releasing gametes, the male and female gametes were collected separately.

Test Solutions Used

Metals were tested for toxicity using the following inorganic salts: $CuCl_2 \cdot 2H_2O$ and $CdCl_2 \cdot 2.5H_2O$. Stock solutions of these metals were made using deionised water and stored in airtight glass bottles. The stock solution of Cu was prepared by diluting 1.0g of $CuCl_2 \cdot 2H_2O$ (Sigma-Aldrich) in 1 litre of water, while the stock solution of Cd was prepared by diluting 0.10g of $CdCl_2\ 2.5H_2O$ (Sigma-Aldrich) in 1 litre of water. The metal stock solutions were then serially diluted with filtered saltwater with salinity of 25% to acquire required concentrations in the test vials the day before testing and allowed to equilibrate overnight. The concentrations tested for Cu were 1ppb, 10 ppb, 100ppb and 1ppm. The concentrations tested for Cd were 0.1ppb, 1ppb, 10 ppb and 100ppb.

Fertilisation Bioassay

The fertilisation bioassay was carried out based on methods modified from those described by DHI, Singapore (DHI, 2008). Four replicates were used for each test concentration

and controls. A further four replicates of unfertilised eggs (unfertilised controls) were included to allow the estimation of the total number of eggs in each test beaker at the start of the test. Tests were conducted in 250 ml beakers and each beaker contained 60 ml of test solution. A volume of 500 µL of egg suspension was added to each test beaker in a randomised order by using 5 pipettes of 100 µL. Before each 100 µL pipetting, the egg suspension was mixed by gentle stirring.

The test was initiated by adding 500 µL of sperm suspension to each test beaker. For the four unfertilised controls, no sperm suspension was added. Instead, 500 µl of 400 mg/l potassium dichromate solution was added. This was to kill the eggs to allow the estimation of the total number of unfertilised eggs in each test beaker at the start of the test. The resulting mixtures were stirred and the test beakers kept in the laboratory.

After 24 hours, the number of unfertilised eggs in each test beaker was counted. Each test beaker was first stirred before pouring the entire test solution out into a glass petri dish. The petri dish was then examined under a dissection microscope and the number of unfertilised eggs in a single field of view (5 mm diameter) was counted. Five counts are made for each test solution under five different fields of view to give an estimate of the total number of unfertilised eggs in a test beaker. This was repeated for all the test beakers. The total number of unfertilised eggs in each test beaker was recorded as a projected estimation of a total area of 180 cm^2 within the petri dish.

Statistical Analyses

Raw data obtained (in terms of percentage of unfertilised eggs) was arcsine transformed before data analysis. Following this, one-way Analysis of Variance (ANOVA) test was carried out on fertilisation success in copper and cadmium test solutions. Dunnett's test was also conducted to assess differences between mean values obtained from control and test solutions. At $P<0.05$, differences were considered to be statistically significant. Probit analysis was then used to obtain EC_{50} values (metal concentration inducing 50% fertilisation). All of the above statistical analyses were carried out using MINITAB (MINITAB, Inc., Release 2007).

Results

The percentage of unfertilised eggs for the different heavy metal concentrations treatments ranged from 9.0 to 26.7 for copper (Fig. 1) and 5.5 to 27.0 for cadmium (Fig. 2). The percentage of unfertilised eggs showed a general increasing trend with increasing concentrations of copper and cadmium. However, corresponding EC_{50} values could not be determined from probit analyses in this study for both toxicants within the range of test concentrations (Fig. 3 and Fig. 4).

Results of one-way ANOVA showed that fertilisation success in controls and treatments for both copper and cadmium treatments were significantly different. According to Dunnett's test, there were no significant differences in fertilisation success between 0.001 ppm, 0.01 ppm and 1 ppm copper and controls (Fig. 1), and there were no significant difference in fertilisation success between 0.0001 ppm and 0.001 ppm cadmium and controls (Fig. 2). Significant effect treatment was observed in all other treatment concentrations as compared to controls (Figs. 1 and 2).

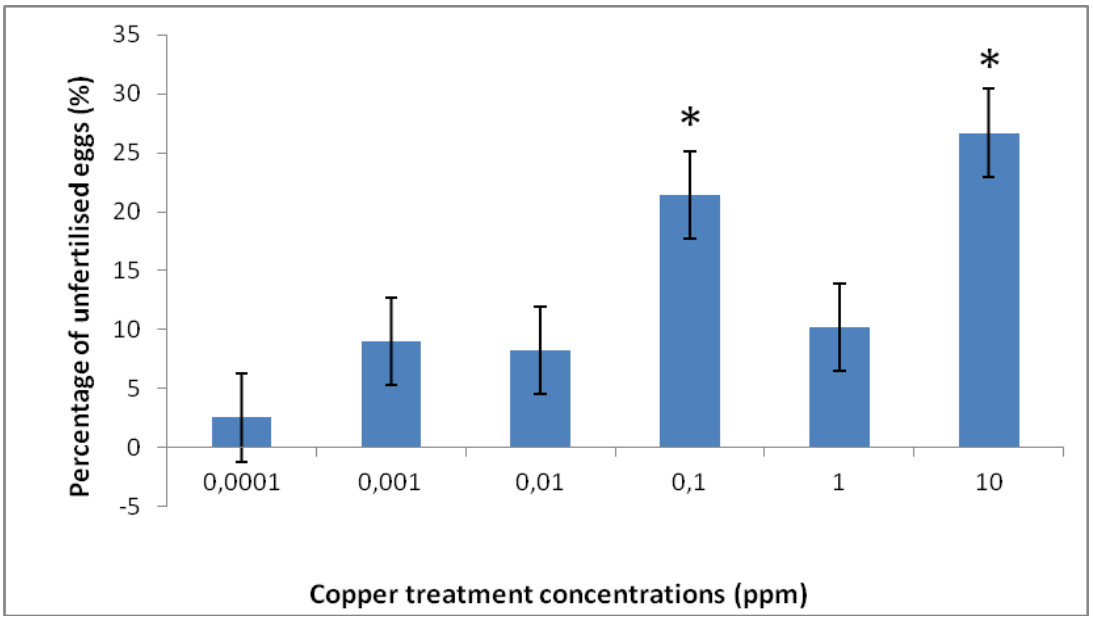

Figure 1. Percentage of unfertilised eggs based on exposure to copper at the different treatment concentrations (ppm: parts per million, mg/l; ***** indicates significant difference in fertilisation success compared to controls in Dunnett's test; $p < 0.05$).

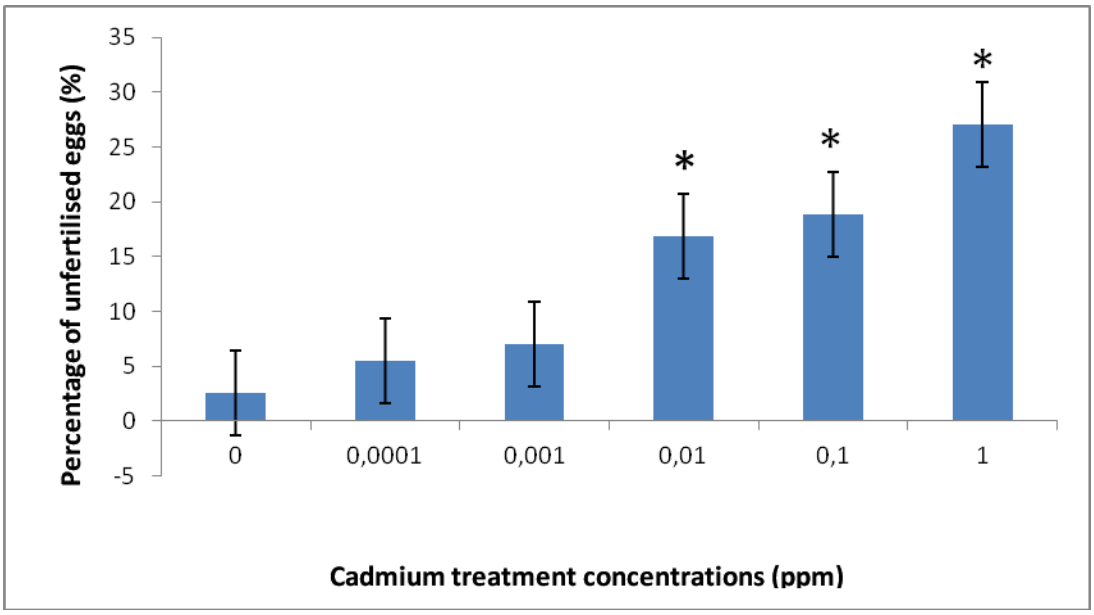

Figure 2. Percentage of unfertilised eggs based on exposure to cadmium at the different treatment concentrations (ppm: parts per million, mg/l; ***** indicates significant difference in fertilisation success compared to controls in Dunnett's test; $p < 0.05$).

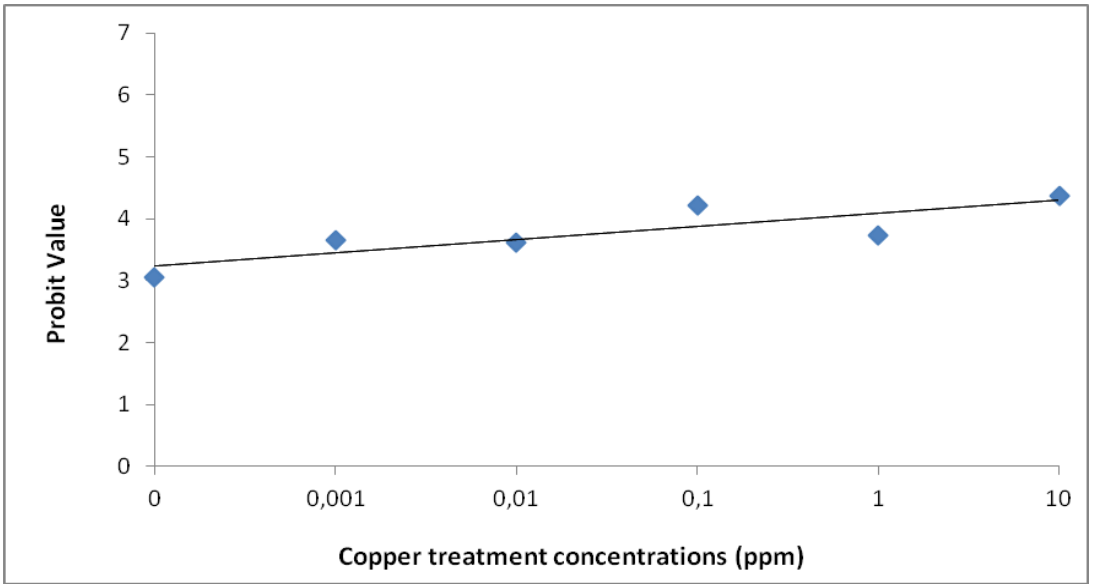

Figure 3. Probit response curve for copper treatment (ppm: parts per million, mg/l).

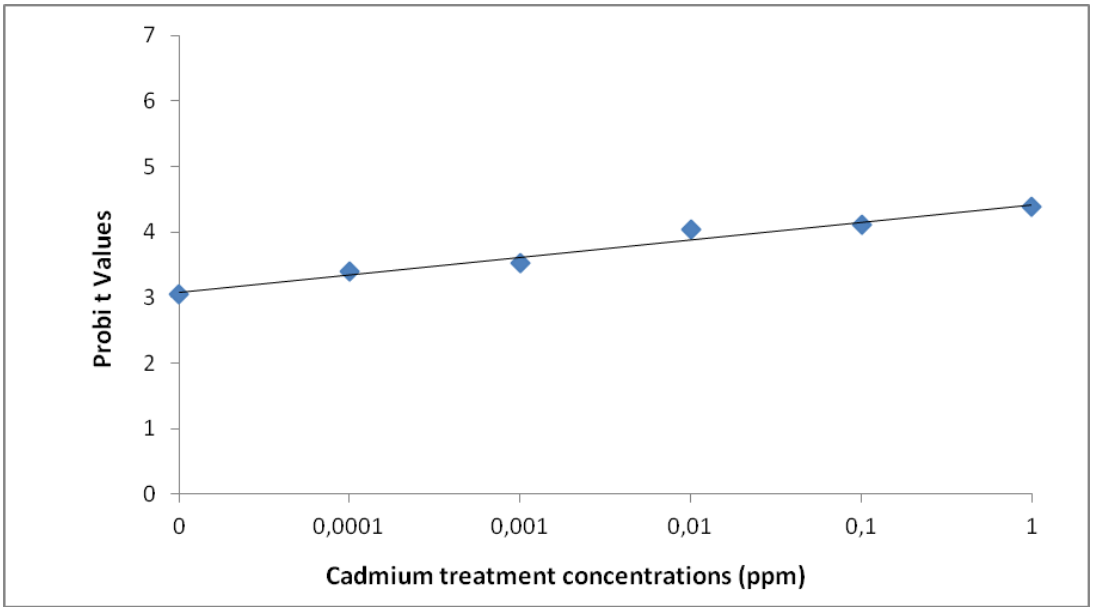

Figure 4. Probit response curve for cadmium treatment (ppm: parts per million, mg/l).

Degenerated unfertilised eggs were observed in both copper and cadmium treatments after the 24 hours exposure (Figs. 5A & B). The amount of degenerated unfertilized eggs observed in the test concentrations of both copper and cadmium increased along with the concentrations of the toxicants. It was also observed that a much lower number of trochophore larvae were present after the 24 hours exposure as compared to the seawater controls (Fig. 5C).

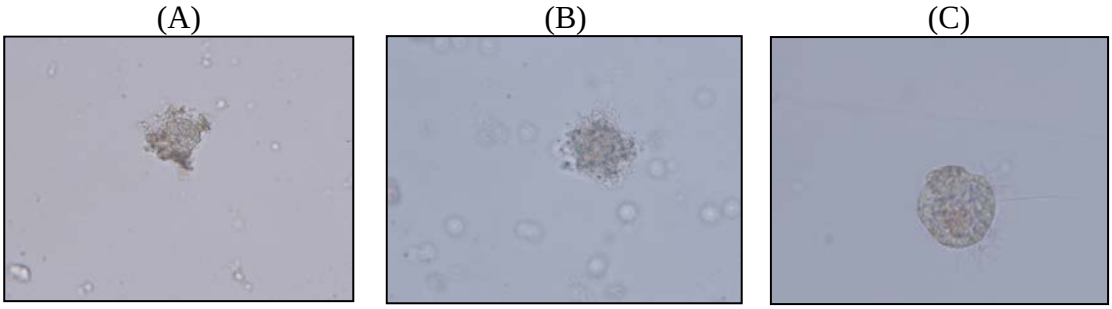

Figure 5. (A) Degeneration of an unfertilized egg observed in 10ppm of cadmium. (B) Degeneration of an unfertilized egg observed in 1ppm of copper. (C) Trochophore larvae observed in seawater control.

Discussion

The results suggest that as copper and cadmium concentrations increased, the percentage of fertilisation success decreased, hence a dose-response relationship between exposure to copper and cadmium and fertilisation in *P. viridis* was able to be established.

The results from the fertilisation experiments showed that copper had little effect on the fertilisation process of *P. viridis* at concentrations up to 0.01ppm of copper and up to 0.001ppm of cadmium respectively, while beyond those concentrations, the toxicants had a negative impact on fertilisation. This indicates that *P. viridis* gametes are more sensitive to cadmium than copper.

However, Ringwood (1992) showed that cadmium concentrations up to 100 µg/l had minimal effect on the fertilisation success of the bivalve species *Isognomon californicum,* whereas the EC_{50} for copper was 55 µg/l for the same species. Nadella *et al.* (2009) also showed that copper was more toxic than cadmium to developing embryos of the blue mussel *Mytilus trossolus* with a 48h EC_{50} value of 9.6 µg/l for dissolved copper and 502 µg/l for dissolved cadmium respectively. These two studies showed that copper was more toxic than cadmium to the respective test organisms, which was a contrasting result to our study. This can be attributed to the varying sensitivities and tolerance between bivalves to copper and cadmium toxicity. Also, differences in experimental procedures and conditions between the studies may have caused the differences in results.

In our study, the presence of debris in the test concentrations was also observed, and that the amount of debris found increased along with the concentration of both copper and cadmium

as compared to the seawater controls. The debris could possibly be degenerated fertilised eggs that did not develop successfully into the free-swimming trochophore larvae. Previous studies on marine invertebrates have shown that prior to fertilisation, eggs are relatively insensitive to acute exposures to heavy metals (Fitzpatrick *et al.,* 2008). However, right after fertilisation, eggs become metabolically active, and membrane ion permeability increases significantly (Franchet *et al.,*1997). This could explain why even at low levels of copper and cadmium concentrations, there were only few free-swimming trochophore larvae observed, despite relatively high percentage of fertilisation.

A study by Fitzpatrick *et al.* in 2008 showed that the rate of fertilisation also decreased when the sperms of the blue mussel *Mytilus trossulus* were exposed to increasing concentrations of copper. Sperms exposed to higher concentrations of copper swam more slowly, and demonstrated reduced fertilisation ability as compared to sperm swimming in seawater control. Similarly, slower sperm swimming speed has been observed in the sea urchin *Anthocidaris crassispina* following exposure to high concentrations (1000–100,000 µg/l) of cadmium (Au *et al.,* 2000). This could explain the decreasing trend in fertilisation in *P. viridis* when exposed to increasing toxicant concentrations observed in our study.

Heavy metals like copper and cadmium may pose high toxicities to marine organisms when introduced into marine ecosystems. (Goldberg, 1995). The levels of heavy metals such as copper and cadmium found in coastal waters are of primary concern because their persistent nature which allows them to bioaccumulate in marine organisms, and be amplified in the food chain, resulting in a threat to the viability of marine organisms' populations and also to human health in the form of seafood (Boening, 1999; Yusof *et al*. 2003; Zhou *et al*. 2008). Human health risks reported due to aquatic metal exposure include abnormal development of fetus, procreation failure and immunodeficiency (Chang *et al.,* 2000). Hence, there is a need for regular monitoring for toxic heavy metal pollutants in water bodies.

The dose-response relationship established in this study provide evidence that fertilisation of *Perna viridis* is sensitive to copper and cadmium, and can be used as an effective biomarker for exposure to these heavy metals. Thus, the *Perna viridis* fertilisation inhibition bioassay is a relevant toxicity bioassay that can be used for monitoring copper and cadmium levels of water bodies.

However, a further study similar to the current study could be conducted to obtain EC_{50} values for copper and cadmium to increase the effectiveness of the *Perna viridis* fertilisation inhibition bioassay as a biomonitoring tool.

Toxicants generally affect organisms at the molecular level by reacting with biochemical substances within cells (Moore, 1985). Thus, further studies could also be conducted to study the molecular effects of the exposure to copper and cadmium in the green mussels, to provide a more comprehensive account of how these toxicants affect green mussels at different organismal levels.

Conclusion

Results obtained confirm a dose-response relationship between copper and cadmium and the number of unfertilised eggs remaining after a 24 h test period. Also, observation under

microscope displayed a degeneration of female gametes upon exposure to the heavy metals. Further studies may be carried out to determine the contributing factors of this relationship at a cellular level. Research detailing the impact of increasing concentrations of copper/cadmium on male and female gametes of *Perna viridis* may also be carried out.

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