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Effect of Heavy Metals on Embryonic Development in the Mussel, *Mytilus galloprovincialis*

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ABSTRACT

The embryos of marine bivalves have been commonly used in bioassays for quality assessments of marine environments. Although several standard protocols for the developmental bioassay of bivalves have been proposed, only a few trials for application of these protocols in environmental assessments or for the development of a new protocol with Korean species have been conducted. As such, there is a strong need to establish standard bioassay protocols with bivalves commonly found in Korean waters. To determine the sensitivity of *Mytilus galloprovincialis* to establish a standard bioassay, their fertilized eggs were exposed to six metals (Ag, Cd, Cr, Cu, Ni, and Zn). The order of biological impact was Ag > Cu > Ni > Zn > Cr > Cd and their lowest observed effective concentration were 5, 16.4, 25.4, 142, 187 and 1,500 μg/l, respectively. The proportion of normal larvae appeared to decrease linearly with the logarithm of each toxicant concentration within the tested range. The average values of median effective concentrations (EC_{50}) from the triplicate experiments for Ag, Cd, Cr, Cu, Ni, and Zn were 6.8, 1,797, 786, 16.6, 68.1, and 139.2 μg/l, respectively. There was a more than 100-fold difference in EC_{50} values of Cu and Cd. The value of EC_{50} or median lethal concentration of Cu was within the range observed for other bivalve developmental bioassays. The overall sensitivity of *M. galloprovincialis* in the present developmental bioassay was also similar to that of other marine organisms commonly used in aquatic bioassays (*e.g.* sea urchins, oysters). Hence, the bioassay using the embryo of *M. galloprovincialis* is considered to be a useful tool to monitor and evaluate the quality of marine aquatic environments.

Keywords: *Mytilus galloprovincialis*, Embryonic development, Bioassay, Sensitivity to metals.

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INTRODUCTION

Bioassays using bivalve embryos have been globally used for the assessment of marine environments. Standard protocols of bioassays utilizing mussels have been elucidated (ASTM, 1994). Mussels are cosmopolitan in nature and easily collectable in coastal areas. In addition, the spawning induction of mussels is relatively effortless by simple physical treatments in the laboratory. Consequently, numerous studies have utilized the developmental stages of mussels for bioassays (His *et al.*, 1996; Jha *et al.*, 2000; Beiras *et al.*, 2003; Nicholson and Szefer, 2003; Beiras and Albentosa, 2004; Kurt and Ozkoc, 2004).

In Korea, the main area of such studies is the aquaculture site of mussels. Studies on mussels have focused only on the larval stage after embryonic development (Hur and Hur, 2000). Meanwhile, environmental studies addressing mussels have mostly focused on chemical analyses such as bioaccumulation and distribution of pollutants. Our previous study (Sung *et al.*, 2005) reported the optimal conditions of salinity, temperature, and embryo density for *Mytilus galloprovincialis* bioassays.

The purpose of the present study is to determine the sensitivity of *Mytilus galloprovincialis* to metals with respect to embryonic development. The fertilized eggs of *M. galloprovincialis* were exposed to six metals (Ag, Cd, Cr, Cu, Ni, and Zn) and effective concentrations of these metals were obtained. The effects of toxic metals on the embryonic development of *M. galloprovincialis* were compared to those of other organisms to verify its utility for bioassay to assess of marine environments.

MATERIALS AND METHODS

In February 2004, mature mussels were collected (shell length: 4-7 cm) at a rocky coast of Jangmok, Geoje Island in Korea. The mussels were then acclimated at 10℃ in a continuous flow system of natural seawater for four weeks to prevent natural spawning. During the acclimation period, they were fed with three kinds of micro-algae, *Isochrysis galbana, Chaetoceros gracilis,* and *Nannochloris oculata.*

The shells of the mature mussels were washed and cleaned with a brush using freshwater to remove adhering organisms like protozoa and other micro animals and weeds.

For spawning induction, mussels were dipped in sea water (20℃, 32 psu) filtered with a 1 ㎛ cartridge after one hour of aerial exposure. The artificially fertilized embryos were passed through a 100 ㎛ nylon mesh to exclude feces and other large particles. The sperms, small particles and retarded embryos were passed several times through a 40 ㎛ nylon mesh until no sperm was observed.

The metals used in the experiments were silver (as $AgNO_3$, 99%), cadmium (as $CdCl_2$, 99%), chromium (VI) (as $K_2Cr_2O_7$, 99%), copper (as $CuCl_2 \cdot 2H_2O$, 99%), nickel (as $Ni(NO_3)_2 \cdot 6H_2O$ 99%), and zinc (as $ZnCl_2$, 98%). All metals were A.C.S. reagent grade and were purchased from Aldrich.

The definitive test concentration range of each metal and median lethal concentration (LC_{50}) values were determined from a range-finding test. All experiments were conducted in triplicate at 15℃ for 48 hr in 20-ml scintillation glass vials (Wheaton).

First, 5 ml of each concentration of six chemicals was prepared in the vials and 500 embryos of *Mytilus galloprovincialis* were added to each vial. After exposure, the embryos were fixed with 10% buffered formalin. Metal concentrations were analyzed using inductively coupled plasma (ICP) at the Korea Basic Science Institute (KBSI).

Quality assurance/quality control was also conducted by conducting the test twenty times with copper as a reference toxicant under the same conditions.

The embryos were examined for their developmental stages and morphological conditions under a stereo-microscope and categorized into four types based on their embryonic developmental stage: 'Dead' - embryos retained at a fertilized egg or early cell division stage 'Retarded' - larva that was hatched but

Table 1. Values of toxicity parameters of *Mytilus galloprovincialis* for different metals.

Metals	Toxicity Parameter		
	NOEC (㎍/L)	LOEC (㎍/L)	LC_{50} (㎍/L)
Ag	1.30	5	98.2
Cd	375	1,500	41,502
Cr	94.0	187	26,118
Cu	13.4	16.4	32.5
Ni	15.4	25.4	-
Zn	116.6	142	-

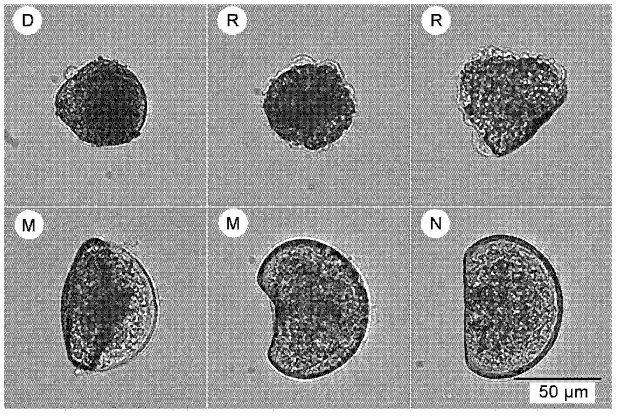

Fig. 1. Different abnormalities and normal larva observed in *Mytilus galloprovincialis*. (D) dead embryo; (R) retarded larva; (M) malformed larva; (N) normal larva.

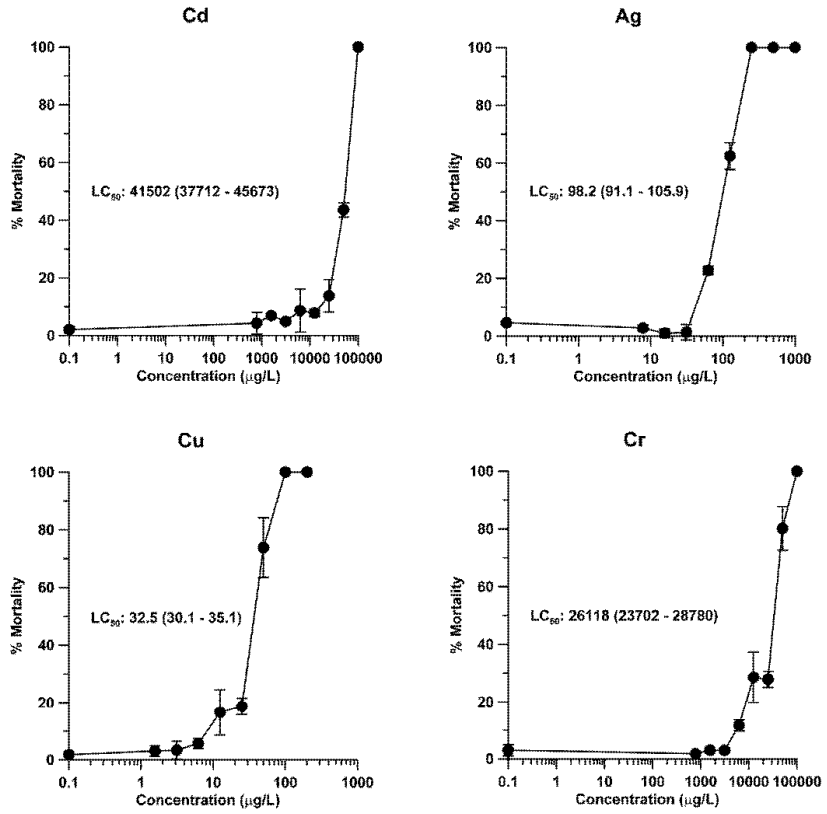

Fig. 2. Concentration-response relationship between metals and the proportions of mortality of *Mytilus glloprovincialis.*

Table 2. Comparison of EC_{50} of *Mytilus galloprovincialis* for different metals.

Metals	EC_{50} (μg/L)				
	Experiment 1	Experiment 2	Experiment 3	Mean ± SD	CV (%)
Ag	5.6	9.8	4.9	6.8 ± 2.7	39
Cd	1,829	1,809	1,786	1,797 ± 16	0.9
Cr	753	794	810	786 ± 29	3.7
Cu	16.8	16.6	16.6	16.6 ± 0.1	0.7
Ni	84.8	55.6	64.1	68.1 ± 15.0	22.0
Zn	138.2	140.5	139.0	139.2 ± 1.2	0.5

not fully developed to veliger 'Malformed' - veliger larva that was deformed or smaller than normal and 'Normal' - larva with a perfectly D-shaped (straight hinge) shell and 90-110 μm shell length. We defined the embryos remained at the fertilized eggs or retarded or deformed or small sized veliger as abnormal and only normal sized veliger as normal (Fig. 1).

The no observed effective concentration (NOEC) and lowest observed effective concentration (LOEC) were determined by Dunnett's t-tests (Sokal and Rholf, 1981). Median lethal concentration (LC_{50}) and median effective concentration (EC_{50}) were estimated by the trimmed Spearman-Karber method (Hamilton *et al.*, 1977) using the TOXSTAT program (Gulley and WEST, Inc., 1996).

For evaluating the fertilization rate, more than 100 eggs were observed under microscope, where the polar body on the egg's membrane was used as an indicator of a fertilized egg. Embryos with a fertilization rate higher than 98% in the brood stock were only used for this work.

RESULTS

Embryos were exposed to Ag, Cd, Cr, Cu, Ni, and Zn in ranges of 0-80, 0-24000, 0-6000, 0-44.4, 0-325.4, and 0-352.6 μg/l, respectively. After exposure, each embryo was examined and designated as normal or abnormal type. NOEC, LOEC, and LC_{50} were estimated from these data (Table 1).

The NOEC of Ag, Cd, Cr, Cu, Ni, and Zn were 1.3, 375, 94, 13.4, 15.4, and 116.6 μg/l, respectively and the LOEC of Ag, Cd, Cr, Cu, Ni, and Zn were 5.0, 1500, 187, 16.4, 25.4, and 142 μg/l, respectively. The LC_{50} values of Ag, Cd, Cr, and Cu were 98.2, 41,502, 26,118, and 32.5 μg/l, respectively (Fig. 2 and Table 1). The LC_{50} values of Ni and Zn could not be estimated because they were not within the range of concentration used in this test.

The EC_{50} of Ag, Cd, Cr, Cu, Ni, and Zn were estimated as 6.8 ± 2.7, 1797 ± 16, 786 ± 29, 16.6 ± 0.1, 68.1 ± 15.0, and 139.2 ± 1.2 μg/l, respectively (Table 2).

The control chart for the embryo toxicity test was obtained by repeated experiments with copper as a reference toxicant (Fig. 3). The average value and standard deviation of EC_{50} were 16.65 ± 1.3 μg/l and the 95% acceptability range (control limit) of EC_{50} was 14.0-19.2 μg/l. The EC_{50} of copper obtained from the sensitivity test in this study was in the range of 16.6-16.8 μg/l, which is within this 95% acceptability range.

DISCUSSION

Comparison of EC_{50} for 6 metal species (Ag, Cd, Cr, Cu, Ni and Zn) in the embryonic development

Table 3. Comparison of EC_{50} values of different species with developmental bioassay of *Mytilus galloprovincialis* commonly used in aquatic toxicity tests.

Species	Exposure duration	EC_{50} μg/l						References
		Ag	Cd	Cr	Cu	Ni	Zn	
Mytilus galloprovincialis	48 h	6.8	1,797	786	16.6	68.1	139.2	This study
Mytilus galloprovincialis	48 h		1,925		10		160-320	Beiras & Albentosa (2004)
Mytilus edulis	48 h				5.8		175	Martin *et al.* (1981)
Crassostrea gigas	48 h	19	1,100		12		207	Dinnel *et al.* (1983)
Crassostrea gigas	48 h				5		119	Martin *et al.* (1981)
Crassostrea gigas	48 h				6		207	Dinnel (1991)
Crassostrea gigas	48 h				9			Coglianese and Martin (1981)
Crassostrea gigas	48 h				14.7			His *et al.* (1999)
Crassostrea iradelei	48 h				81			Ramachandran *et al.* (1997)
Ruditapes decussatus	48 h		424		9.1		129	Beiras and Albentosa (2004)
Haliotis rufescens	48 h				9		68	Hunt and Anderson (1989)
Strongylocentrotus purpuratus	96 h	15	510		6		23	Dinnel *et al.* (1989)
Strongylocentrotus purpuratus	96 h				11			Bay *et al.* (1993)
Paracentrotus lividus	72 h		200	3,100	60	320	49	Volpi Ghirardini *et al.* (2003)
Arbacia punctulata	4 h	179	13,900		14		205	Nacci *et al.* (1986)
Scylla seratta	48 h				80			Ramachandran *et al.* (1997)
Ciona intestinalis	20 h		839	10,296	46			Calabrese *et al.* (1977)

bioassay showed that the sensitivity of the mussel *Mytilus galloprovincialis* to the metals was in the following order: silver > copper > nickel > zinc > chromium > cadmium. That is, this species was more sensitive to silver than the other five metals in this study (Table 2).

The mortalities to toxicants used in the definitive test were lower than 20% at the highest concentration. The proportions of retardation and malformations varied among various toxicants at the highest concentration (Fig. 4). In the case of silver, the malformation rate was 3.8-fold higher than the retardation rate. In contrast, the retardation rate for zinc was 3.6-fold higher than the malformation rate. The responses of developing embryos were different for each toxicant. Generally, some of metals in the tissues are involved in the regulation of gene networks. Exposure to excess amount of metals may alter gene expression profiles in the target tissues/cells. The metal can also combine with certain proteins, which can transform their characteristic structures and promote or inhibit their functions. This could be the reason that the embryos exposed to metal experience growth retardation or malformation (Irwin *et al.*, 1997).

We compared *Mytilus galloprovincialis* with other organisms used commonly in aquatic bioassays (EC_{50} or LC_{50}) (Table 3). The EC_{50} values of *M. galloprovincialis* to metals were at a comparable level with those of other marine organisms and the values were close to the oyster, *Crassostrea gigas* (His *et al.*, 1999).

Mytilus galloprovincialis inhabits all coasts of Korea. Five to twenty million eggs can be synchronously harvested by spawning of a matured mussel (Choe *et al.*, 1999). This can be advantageous in that many experiments can be conducted from a single spawning. The main spawning season of the mussel is between February and May in Korea, but we could obtain embryos by a simple physical treatment, even in periods other than the spawning season. The optimum conditions to conduct embryonic development tests were established in a previous study (Sung *et al.*, 2005).

This study was the first trial in Korea on embryonic development bioassay using *Mytilus galloprovincialis.* This mussel showed acceptable sensitivity as compared with other marine organisms for bioassays. Accordingly, it can be considered that can be a better species for conducting bioassay experiments. Bioassay

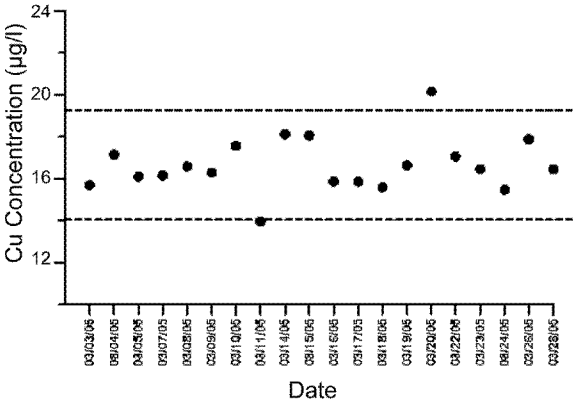

Fig. 3. Control chart for embryo toxicity test obtained by repeated experiments with the reference toxicant (Copper). Dotted lines: acceptable range.

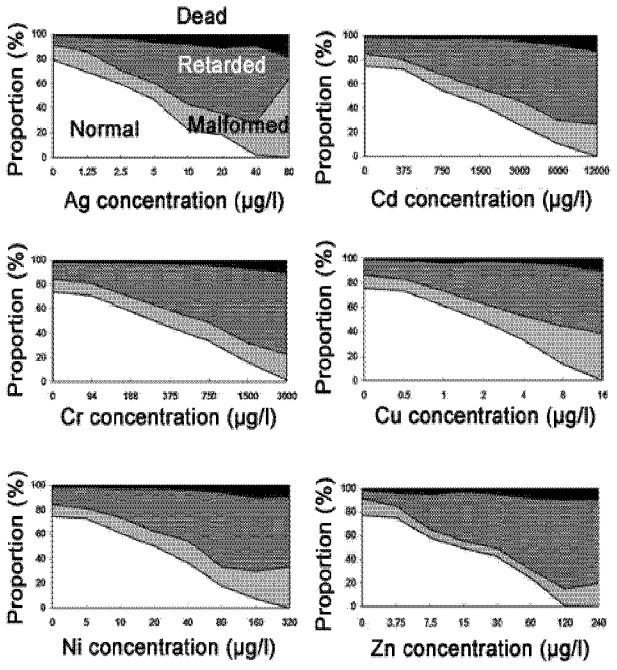

Fig. 4. Comparison of proportion of each developmental stage of *Mytilus* at different concentrations of metals.

using *M. galloprovincialis* embryo had perceptible sensitivity to metals such as copper, which affect organisms even at extremely low concentrations. The information on embryonic development of the Korean mussel, *M. galloprovincialis,* garnered through this study may be efficiently utilized for standard protocols for the bioassay.

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