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Original Research Article

Acute Toxicity of Copper in Freshwater Molluscs *Lamellidens corrianus, Parreysia (Parreysia) gowhattensis and Melania hainesiana* and Effect on Behavior

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Abstract: A study was carried out to observe the behavioral changes in three freshwater molluscs species *Lamellidens corrianus* (Lea, 1834), *Parreysia (Parreysia) gowhattensis* (Theobald, 1873), and *Melania hainesiana* (Lea, 1856) due to copper (Cu) exposers. Firstly, acute toxicity was carried out to select the exposed concentrations of Cu. After the exposure in Cu test concentrations, Cu was found to be accumulated in the tissues of mollusc. Kruskal-Wallis test of behaviors showed significant differences among the control and test concentration of Cu. So, the null hypothesis was rejected; i.e., the distribution of movement, visibility of only siphons extension, extension of foot along with visibility of siphons together at the same time and closing of valves without extension of foot and siphon of *L. corrianus, P. (P.) gowhattensis* were not the same across control, 1% and 10% of 96 h LC_{50} of Cu. A Kruskal-Wallis test also revealed that in *Melania hainesiana* significant differences in crawling speed and duration of foot-protrusion among control, 1% and 10% Cu exposed concentrations.

Key words: Behavior, Bivalves, Copper, Gastropod, Toxicity

Introduction

The term environmental toxicology is a multidisciplinary science under which the study of the harmful effects of numerous chemical, biological and physical substances on living organisms are practiced [URL-1] [URL-2]. In biological point of view, behavior is the internally coordinated responses (actions and inactions) of whole living organisms (individual or groups) to internal and/or external stimuli (Levitis *et al.* 2009). Behavior contributes outputs from the organisms to the environment (Dusenbery 2009). To understand behavior, we must know the cause that may be external or internal stimuli, processes, contingencies, and ontogeny or the behavior developed in a lifetime due to interaction of genetic and environmental factors (Tinbergen 1963). Baum (1974) suggested that laboratory tests derived from experimental analyses of behavior could be adapted for use in the natural environments. Kendall (1982) has stated that ecotoxicology would be benefitted by utilizing the approaches "attempt to recognize elements of the animal's own behavior and to identify situations where they occur reliably enough for experimental use" (Silverman 1988), and "the identification of functional units of behavior, under which they reliably occur, and the variables which modify their occurrence" (Skinner 1974).

Aquatic molluscs have been used as a suitable organism in both active and passive biomonitoring (Goldberg 1986; Salanki 1989; Borcherding and Volpers 1994). In passive monitoring level of pollution of the ecosystem, effect of pollution at the level of populations by elimination of sensitive species and reduction in biodiversity and at the individual level accumulation of pollutants in the organs and tissues are detected, whereas, active monitoring includes response of the artificial or modified populations, behavioral patterns, movement, feeding, respiration, reproduction, neural regulation, cellular and sub cellular functions under toxic conditions (Salanki *et al.* 2003).

Behavioral endpoints are thousands of times more sensitive than the median lethal concentration (LC_{50}) (Hellou *et al.* 2008; Robinson 2009). Identifiable behavioral changes in an organism hints the changes in the environment and these behavioral endpoints comprising different activities may be ranked according to the time, recognizing it as "early warning" (Clotfelter *et al.* 2004).

Advantage of studies based on growth and reproduction is considered more precise, but the disadvantage lies in the longer duration of expose time, specifically in vertebrates, whereas, behavioral endpoints have shorter exposure times and very sensitive (Melvin and Wilson 2013). Study of ecotoxicology by using behavioral parameters is simple but effective, low cost, and specifically effect on feeding behaviors is known to be ecologically significant (Alonso *et al.* 2016). Hartmann *et al.* (2016) introduced a scientific method to use freshwater mussel behavior, such as filtration, valve transition frequency, and avoidance, to be eco-toxicological endpoints for various physicochemical changes and contaminations. The molluscs used in this study are abundantly found in this region. The use of invertebrates in the laboratory in ecotoxicology is preferred, because their maintenance in laboratory is cheaper and includes lesser ethical involvements (Alonso *et al.* 2016). Molluscs are sensitive to different types of toxicants and proved as a good bioindicator in biomonitoring (Tallarico 2015).

The "Hazardous Metals" also termed as "Trace Elements" is used in geochemical and biochemical literature refer to a group of otherwise unrelated chemical elements which are found in nature at very low concentrations. In many cases, metals occur in natural water bodies at levels below their toxic thresholds (WQAA 2014). However, due to their non-degradable nature, even such low concentrations may pose risk of damage via uptake and subsequent bioaccumulation by organisms, which cannot effectively metabolize and excrete the absorbed metals. Several scientific observations have shown that heavy metals are bio-concentrated or bio-accumulated in one or several compartments across food webs (Soegianto and Irawan 2008; Celechovska *et al.* 2008). Besides, contamination of resources with trace elements may have devastating effect on natural ecosystem functioning, as well as decrease of biodiversity and extinction of sensitive taxon (Bogatov and Bogatova 2009; Bonanno and Giudice 2010).

Copper (Cu) is an essential element to most life forms. However, at high bioavailable concentrations, Cu becomes toxic. With its application in industry and agriculture (e.g., Cu containing fungicides and herbicides), Cu release from these sources into the environment is substantial (Alva *et al.* 1995; US DA 2006; Hoang *et al.* 2008). In Asia, recent industrial and economic development has increased pressure to the environment. Cu^{2+} ions are known to be particularly toxic to a wide variety of aquatic organisms (Anderson and Morel 1978; Toledo *et al.* 1979; Brown and Rattigan 1979; Muramoto 1982; Tanaka *et al.* 1982; Zamada and Sunda 1982; Andrew *et al.* 1977; Swartzman *et al.* 1990; Xue and Sigg 1990). No behavioral tests have been standardized and few have been verified in the field (Atchison 1987). The toxicant causes stress on the organisms; the behavioral changes are the immediate responses to the toxicant and are indicators of possible stress (Ait *et al.* 2011). Increased mucus secretion and decrease in shell closure responsiveness of the freshwater mussel may be used as biomarker for the assessment of actual health of the organisms living in the polluted water (Kumar *et al.* 2012).

Molluscs have long been regarded as promising bioindicators and biomonitoring subjects. In present research work a trial was being made to evaluate the toxicity of metal copper on the selected mollusc species viz, *Lamellidens corrianus* (Lea 1856), *Parreysia (Parreysia) gowhattensis* (Theobald 1873) and *Melania hainesiana* (Lea 1856). These three molluscs were selected for our research work as they are found abundantly in the sampling sites of Kokrajhar District, Assam, India and many communities use them as food. The behavioral changes observed during the behavioral tests to examine their sensitivity compared to that of normal behavior in control in the laboratory condition would be reported.

Materials and methods

Procurement of copper

The metal used in the present study was Cupric Chloride, Molecular formula: $CuCl_{2.}2\ H_2O$., manufactured by Thermo Fisher Scientific India Pvt. Ltd., Mumbai, India.

Collection and acclimatization of test organisms

In present work the sensitivity of three freshwater mollusc species to copper: *L. corrianus*, *P. (P.) gowhattensis* and *M. hainesiana* were tested. Molluscs were handpicked directly from the muddy sediments of river Samoka, Kokrajhar, Assam, India. The upstream part of river Gaurang is called Samoka. Collection was made from a single station and approximately same sizes were selected so the result does not vary during the tests. The selected sizes for test were: *L. corrianus* of length, breadth, weight 65.13 ± 3mm, 2.2 ± 1.1 mm, 25.03 ± 5.3 g; *P. (P.) gowhattensis* of length, breadth, weight 23.4 ± 2.1 mm, 17 ± 1.2 mm, 7.2 ± 0.23 g respectively. *M. hainesiana* of length 30.3 ± 1.2 mm, and weight 2.12 ± 0.5g were collected from the Samoka, Kokrajhar, Assam, India during December-March, 2022. They were handpicked from the base of stream which is a mix of sand, small pebbles and mud, at around 7:00 - 9:00 a.m. The collected live molluscs were brought in a plastic bucket with some stream water in it. The species were identified by using the taxonomic keys given in the Handbook of Freshwater Molluscs of India (Subba Rao 1989). The collected species were brought to the laboratory and reared in glass aquaria (60 x 30 x 30) cm of 40L capacity and acclimatized for about 7 days prior to the experiment in the laboratory condition. Aquarium was covered with nylon net so that any snail could not crawl out of it. The following conditions were followed during the acclimatization: feeding was avoided to empty the gut content, aeration was provided

Lamellidens corrianus

Parreysia (Parreysia) gowhattensis

Melania hainesiana

continuously, water was renewed every day, and dead animals if any were removed to prevent water from fouling.

Water quality

The water qualities of the test medium were measured: temperature with a mercury bulb thermometer, pH with a digital pH meter, electric conductivity with a conductivity-TDS meter. Dissolved oxygen (DO), free CO_2, alkalinity, total hardness were recorded (APHA et al. 2005) at the beginning of the experiment, before the animals were exposed in the test chamber i.e., at 0 h.

Acute Toxicity

After acclimatization, a range finding test was conducted with two selected concentrations before the definitive test which enables to know the appropriate range of concentrations. A static-with-renewal 96 h bioassay test was performed with renewal of test solution every 48 h. Groups of experimental organisms consisting of 7 individuals each were selected at random and used in the control (zero metal concentration) and each metal treatment concentrations. Food was withheld one day prior to the experiment, and the test organisms were not fed during the experiment. Similarly, controls were also maintained in which no metal was added. Mortality was recorded at 24, 48, 72 and 96 h, and dead organisms if any, were removed (OECD 1992). The end points of mortality in acute toxicity test in bivalves: no movement, discharge of large amount of milky white substance, valves remain open when mantle margin was mechanically stimulated, foot extensively remains extended outside the shell and float on the surface of the solution (Brahma and Gupta 2020). The end points of mortality in gastropod: absence of movement or crawling, release of thick milky white mucus, release of soft body from the shell without tightly closed operculum, sometimes float on the surface, and no response to mechanical stimuli.

Molluscs were exposed to 4 concentrations of each metal plus a control as follows: *L. corrianus* in nominal concentrations of 100, 180, 320, 560 mgL^{-1} $Cucl_2.2H_2O$ with measured concentrations of 5.8, 10.44, 18.56, 32.48 Cu mgL^{-1} respectively; *P. (P.) gowhattensis* in nominal concentrations of 2, 4, 6, 8 mgL^{-1} $Cucl_2.2H_2O$ with measured concentrations of 0.116, 0.232, 0.348, 0.464 Cu mgL^{-1} respectively, and *M. hainesiana* in nominal concentrations of Cu (0.744, 2.232, 3.72, and 5.208 mgL^{-1}) with respective measured concentrations (0.115, 0.347, 0.646, and 1.071 mgL^{-1}).

The median lethal concentration (96 h LC_{50}) and its 95% confidence interval (95% CI) for all the test species were estimated by log-probit analysis according to Finney (1971) with the help of SPSS 20 statistical software for windows. Safe concentrations of the metals were determined by the methods of Kameswara-Rao (1974) and Miller & Miller (1986) following the application factor, 1/100th of the 96 h LC_{50} values.

Metal accumulation in experimental animals

Three animals of each species from control and exposed experimental were dissected on 5th day of exposure period and the whole soft body mass of each animal was dried in oven 70 - 80°C. The weight of the whole body is taken after complete oven dried. 500 mg dry weight whole soft body tissue of control and exposed experimental bivalves were digested in concentrated HNO_3 and $HClO_4$ in (5:1) ratio and re dissolved in 10 ml of 10% HNO_3. After cooling double distilled water was added to make the volume to 50 ml. The solution was filtered and Cu and Pb metals were quantified in an Atomic absorption spectrophotometer (AAS) (Perkin Elmer AA 200, model 2005).

Study of behavior

Study of behavioral changes were done by following the protocol of Brahma and Gupta (2020) and experiment was done by exposing the bivalve molluscs in the concentration of 10% and 1% of LC_{50} (median lethal concentration) value at 96 h. A control was also kept for observation. A single animal was exposed in the test media in glass aquarium of (45 x 30 x 30) cm of 40L in the laboratory. The animal was observed continuously for duration of 30 minutes for five hours a day. There was a gap of 5 minutes between every two observations. The observation was continued for five days for each concentration without any disturbance to the animal during the exposure. The observation was done manually as eye observation as well as with the help of camera video recording. No electrical devices were attached to the animal during the observation. In our observation we selected the following parameters as changes in behaviors due to toxicity viz., locomotion behavior (No. of times of movement), visibility of only siphons extension (sec) and extension of foot along with visibility of siphons both together at the same time (sec) and closing of valves without extension of foot and siphon (sec).

To observe the particular behavioral changes as in crawling speed (mm/sec) in *M. hainesiana*, the protocol of Dalesman and Lukowiak (2010) with slight modifications was followed. In addition to this, another parameter i.e., duration of head-foot protrusion (sec) was added.

The concentrations used in this study were 1% and 10% of 96 h LC_{50} values of Cu. Similarly, controls were also kept in tap water without added Cu.

In a Petri dish of size 14 cm diameter by 2 cm depth, 200 ml of test medium containing the given concentrations (Table 2 and 3) was put which fills a depth of 15 mm in Petri dish. This amount of water could submerge the snail completely. One snail was observed at a time. The moment snails were placed in the Petri dish with the media they tried to withdraw the body inside the shell. So, the quantifying of behavior changes started when the snails emerged again. Petri dish was placed over a graph paper with markings of a 2 cm x 2 cm grid. This made the counting of the distance covered by the snail. The observation was done for 15 minutes and recorded with the help of a camera. Along with the crawling distance the head-foot protrusion was also observed. The mean crawling speed was calculated in mmS^{-1}. Total number of observations in each concentration was 10. The observations were started at 11:00 a.m. The observations were done at room temperature of 23-26°C.

Mathematical calculations and Statistical analysis

Crawling speed was calculated by using the equation,

Speed= distance travelled/time, units in mm/sec.

One-sample Kolmogorov-Smirnov Test was used to check the normality of the data. Variance was checked by using Kruskal-Wallis Test and Mann-Whitney statistical analysis. Mathematical calculations and statistical analyses were done in Microsoft Excel 2016 and IBM SPSS Statistics 20 for Windows.

Results

Toxicity test

The physicochemical properties of the test medium were: temperature 16- 18°C; p H 5.4 – 6.5; electrical conductivity 70.2 µS/cm; dissolved oxygen (DO) 8.34 – 10.93 mg L^{-1}; alkalinity 5 – 6 mg L^{-1}; total hardness 8 – 10 mg L^{-1}; free CO_2 1.2– 1.8 mg L^{-1}.

Acute toxicity

The median lethal concentrations (LC_{50}) of copper exposed under static bioassay tests (96 h) of freshwater bivalves *L. corrianus*, *P. (P.) gowhattensis, M. hainesiana* are given in Table 1.

Table 1. Acute lethal toxicity bioassays with copper for freshwater bivalves *L. corrianus*, *P. (P.) gowhattensis* and *M. hainesiana* exposed under static bioassay tests (96 h) .

Metal	Species	LC_{50}(mgL^{-1})	95%Confidence Interval (LB–UB)	Safe Conc. (μgL^{-1})
	L. corrianus	8.336	7.326 – 9.387	83.36
	P. (P.) gowhattensi	0.143	0.113 – 0.167	1.43
	M. hainesiana	0.321	0.1483 – 0.696	3.21

The $LC_{10,}$ $LC_{25,}$ $LC_{50,}$ LC_{75} and LC_{90} values of Cu for *L. corrianus* at 24, 48, 72 and 96 h were found to range from 22.010 – 119.942, 6.192 – 71.781, 5.338 – 27.646 and 5.206 – 13.347 mg L^{-1}, respectively. The median lethal concentration (LC_{50}) values of Cu at 24, 48, 72 and 96 h for *L. corrianus* were found to be 51.381, 21.083, 12.148 and 8.336 mg L^{-1}, respectively.

The $LC_{10,}$ $LC_{25,}$ $LC_{50,}$ LC_{75} and LC_{90} values of Cu for *P. (P.) gowhattensis* at 24, 48, 72 and 96 h were found to range from 0.315 – 1.512, 0.166 – 0.941, 0.080 – 0.543 and 0.070 – 0.290 mg L^{-1}, respectively. The median lethal concentration (LC_{50}) values of Cu at 24, 48, 72 and 96 h for *P. (P.) gowhattensis* were found to be 0.690, 0.395, 0.208 and 0.143 mg L^{-1}.

The $LC_{10,}$ $LC_{25,}$ $LC_{50,}$ LC_{75} and LC_{90} values of Cu for *M. hainesiana* at 24, 48, 72, and 96 h were found to range from 5.467 – 118.13, 0.374 – 5.487, 0.817 – 1.947 and 0.148 – 0.696 mg L^{-1}, respectively. The median lethal concentration

Table 2. Concentration of Cu used in the behavioral study in *L.corrianus*, *P. (P.) gowhattensis* and *M. hainesiana*.

Species	Test group	Nominal Cu concentration (mgL^{-1})	Measured Cu concentration (mgL^{-1})
L. corrianus	Control	0	bdl
	1% 96 h LC_{50}	0.0847	0.008322±0
	10% 96 h LC_{50}	0.8473	0.084221±0.1
P. (P.) gowhattensis	Control	0	bdl
	1% 96 h LC_{50}	0.0010	0.000096±0
	10% 96 h LC_{50}	0.0102	0.00099±0.01
M. hainesiana	Control	0	bdl
	1% 96 h LC_{50}	0.0011	0.000116±0
	10% 96 h LC_{50}	0.0119	0.00107±0.01

Values are in Mean±SD (n=5); bdl: below detection limits.

(LC_{50}) values of Cu at 24, 48, 72 and 96 h for *M. hainesiana* were found to be 25.413, 1.432, 1.261 and 0.321 mg L^{-1}.

Cu in the animal after exposure in 1% and 10% of 96 h LC_{50} of Cu

Cu accumulates in the tissues of bivalves during the experimental exposure. The amount of accumulation varies among the species and also according to the size. The mean (SD) metal concentration (μgg^{1}) in molluscs species *L. corrianus* and *P. (P.) gowhattensis* after exposure to 1% and 10% of 96 h LC_{50} of Cu for 5 days are given in the Table 3.

Table 3. Mean (SD) metal concentration (μgg^{1}) in molluscs species after exposure to 1% and 10% of 96 h LC_{50} of Cu for 5 days.

Metal	*L.corrianus*		*P. (P.) gowhattensis*	
	1%	10%	1%	10%
Cu	20.773 (2.604)	140.093 (7.679)	26.626 (5.425)	2.633 (0.568)

Behavior

Kruskal-Wallis test of behaviors among control, 1% and 10% of 96 h LC_{50} of Cu exposed shows that the differences were statistically significant (H=25.183, p=0), which is less than 0.05. So, reject the null hypothesis; i.e., The distribution of movement, visibility of only siphons extension, extension of foot along with visibility of siphons together at the same time and closing of valves without extension of foot and siphon of *L. corrianus*, *P. (P.) gowhattensis* is not the same across the control, 1% and 10% of 96 h LC_{50} of Cu.

Mann-Whitney test of behavior parameters such as; locomotion behavior movement, visibility of only siphons extension, extension of foot along with visibility of siphons together at the same time and closing of valves without extension of foot and siphon of *L. corrianus* and *P. (P.) gowhattensis* between control and 1% of 96 h LC_{50} and between control and 10% of 96 h LC_{50} of Cu exposed shows that the differences were statistically significant (U=8.000, p=0.001), which is less than 0.05. So, reject the null hypothesis; i.e., The distribution of behavior parameters are not the same between control and 1% of 96 h LC_{50} and between control and 10% of 96 h LC_{50} of Cu concentration.

Locomotion behavior movement of *L. corrianus* and *P. (P.) gowhattensis* in case of 1% and 10% of 96 h LC_{50} of Cu exposed shows that the difference was statistically not significant (p=0.145and p=0.718), which is greater than 0.05. So, retain the null hypothesis; i.e., the distribution of movement is the same between 1% and 10% of 96 h LC_{50} of Cu concentration.

Visibility of only siphons extension of *L. corrianus* and *P. (P.) gowhattensis* in case of 1% and 10% of 96 h LC_{50} of Cu exposed shows that the difference was statistically not significant (p=0.859 and p=0.942) which is greater than 0.05. So, retain the null hypothesis; i.e., the distribution of visibility of only siphons extension is the same between 1% and 10% of 96 h LC_{50} of Cu concentration.

Extension of foot along with visibility of siphons together at the same time of *L. corrianus* and *P. (P.) gowhattensis* in case of 1% and 10% of 96 h LC_{50} of Cu exposed shows that the difference was statistically not significant (p=0.317 and p=0.317) and (p=1.000 and p=1.000), which is greater than 0.05. So, retain the null hypothesis; i.e., the distribution of extension of foot along with visibility of siphons together at the same time is the same between 1% and 10% of 96 h LC_{50} of Cu concentration.

Closing of valves without extension of foot and siphon of *L. corrianus* and *P. (P.) gowhattensis* in case of 1%

and 10% of 96 h LC_{50} of Cu exposed shows that the difference was statistically not significant (p=0.899 and p=0.942), which is greater than 0.05. So, retain the null hypothesis; i.e., the distribution of closing of valves without extension of foot and siphon is the same between 1% and 10% of 96 h LC_{50} of Cu concentration.

Average crawling speed and duration of head-foot protrusion of *M. hainesiana* in control and 1% and 10% Cu groups are tabulated in Table 4 and 5.

Table 4. Duration of head and foot protrusion in *M. hainesiana*.

Metal	Test group	Duration of head and foot protrusion (sec)	Minimum (sec)	Maximum (sec)
Cu	Control	900±0$_a$	900	900
	1%	119.7±30.61$_b$	0	286
	10%	188.3±45.32$_b$	0	428

Table 5. Crawling speed of *M. hainesiana*.

Metal	Test group	Crawling speed (mm/sec)	Minimum speed (mm/sec)	Maximum speed (mm/sec)
Cu	Control	0.292±0.05a	0	0.561
	1%	0.001±0b	0	0.013
	10%	0.005±0b	0	0.014

1%: Cu concentration of 1% of 96 h LC_{50} value, L. corrianus = 0.0847 mgL^{-1}; P. (P.) gowhattensis = 0.001 mgL^{-1}; 10 %: Cu concentration of 10% of 96 h LC_{50} value, L. corrianus = 0.8473 mgL^{-1}; P. (P.) gowhattensis= 0.0102 mgL^{-1}; Mov: movement; FSi: extensive extensions of foot and siphon together.

A Kruskal-Wallis Test revealed that in *M. hainesiana* there were significant differences in crawling speed and duration of foot-protrusion among control, 1%, and 10% Cu exposed concentrations (H=18.272, p=0; and H=14.724, p=0.001).

Mann-Whitney Test revealed that crawling speed and head-foot protrusion in control was significantly different from that in 1% and 10% Cu exposed groups (U=5.500, p=0; U=7.000, p=0.001).

A Kruskal-Wallis Test revealed that there were significant differences in crawling speed and duration of foot-protrusion among control, 1%, and 10% Cu exposed concentrations (H=18.272, p=0; and H=14.724, p=0.001). Mann-Whitney Test revealed that crawling speed and head-foot protrusion in control was significantly different from that in 1% and 10% Cu exposed groups (U=5.500, p=0; U=7.000, p=0.001), while between 1% and 10% Cu exposed groups no significant differences were recorded (U=34.500, p=0.123).

Discussion

Based on 24, 48, 72 and 96 h LC_{50} values, copper was found to be toxic metal to all the tested molluscs species. Order of sensitivity of molluscs to metal copper was found as, *P. (P.) gowhattensis* > *M. hainesiana* > *L. corrianus*. Patil and Mahajan (2012) found the 24, 48, 72 and 96 h LC_{50} of cupric chloride for *L. corrianus* to be 1.95, 1.85, 1.69 and 1.4 mg L^{-1} and those for cupric sulphate to be 1.98, 1.95, 1.92 and 1.67 mg L^{-1}. These values are lower than the 24, 48, 72 and 96 h LC_{50} values for *L. corrianus* found in the present study, which are 51.381, 21.083, 12.148 and 8.336 mg L^{-1}, respectively. On the contrary, 24, 48, 72 and 96 h LC_{50} values of Cu for *P. (P.) gowhattensis* are 0.690, 0.395, 0.208 and 0.143 mg L^{-1} respectively, which are lower than those found for *L. corrianus*, indicating higher sensitivity of *P. (P.) gowhattensis.*

Behavioral parameters shown by the molluscs in 1% and 10% of 96 h LC_{50} Cu exposed highly differ from the control. The effect of metals is seen to have occurred even at the very low concentration (or at safe concentration). The behavioral changes in organisms due to metal exposure indicate that the animals try to avoid the toxic metals in the water (Brahma and Gupta, 2020). The behavioral activities of organisms negatively correlate with the increase of metal concentration. Effect of toxicity was observed in Cu-exposed *L. corrianus* and *P. (P.) gowhattensis* as the first surceasation of movement and extensive extensions of foot and siphon together (Table 6). In the control there was no surceasation of Mov and FSi in, *L. corrianus* and *P. (P.) gowhattensis*. In *P. (P.) gowhattensis* in 1% of 96 h Cu concentration surceasation of Mov was recorded on Day 3 at 2nd h and surceasation of FSi on Day 1 at 1st h, and in 10% of 96 h LC50 Cu concentration surceasation of Mov was recorded on Day 1 at 1st h and

Table 6. Behavioral changes observed in Cu-exposed *L. corrianus*, *P. (P.) gowhattensis*: Surceasation of movement (Mov) and extensive extensions of foot and siphon together (FSi)

Name of species	Treatment	Effect observed	
		Surceasation of Mov	Surceasation of FSi
L. corrianus	Control	Not recorded	Not recorded
	1%	Day 2 at 3rd h	Day 1 at 3rd h
	10%	Day 2 at 1st h	Day 1 at 3rd h
P. (P.) gowhattensis	Control	Not recorded	Not recorded
	1%	Day 3 at 2nd h	Day 1 at 1st h
	10%	Day 1 at 1st h	Day 1 at 1st h

1%: Cu concentration of 1% of 96 h LC_{50} value, L. corrianus = 0.0847 mgL^{-1}; P. (P.) gowhattensis = 0.001 mgL^{-1}; 10 %: Cu concentration of 10% of 96 h LC_{50} value, L. corrianus = 0.8473 mgL^{-1}; P. (P.) gowhattensis= 0.0102 mgL^{-1}; Mov: movement; FSi: extensive extensions of foot and siphon together.

surceasation of Fsi on Day 1 at 1st h. The observation of behavioral changes as surceasation of the movement and extensive extensions of foot and siphon together could give good insight about the effect of very low Cu concentrations 0.0847 mgL^{-1} and 0.8473 mgL^{-1} in *L. corrianus.* Central Water Commission, India has listed Dhadher River of Pingalwada with highest Cu concentration in water 314.930 ìgL^{-1} in the year 2017 and Brahmaputra River of Assam at the site Tezpur with Cu concentration in water 54 ìgL^{-1} in the year 2017 (CWC 2018), therefore, the concentrations which was used in the exposures were much lower than the Cu concentrations which could be recorded in the natural waters of India. There occurs much possibility that the molluscs studied must have struggled to survive and possess normal behavioral in the water containing toxic substance like copper metal in the water. The behavioral changes were also observed in *M. hainesiana* in exposure to 1% and 10% of 96 h LC_{50} (0.0011 and 0.0119 Cu mgL^{-1}). Speed was observed to be significantly reduced in both the Cu concentrations compared to that of control. The reason of reduced speed of crawling might be due to the reduced tentacle movement with withdrawing in of the soft body inside the shell to escape the toxic concentration of copper metal in the water. Significant positive correlation of movement with extensive extensions of foot and siphon together was recorded in *L. corrianus* and *P. (P.) gowhattensis.* Movement and extensive extensions of foot and siphon were both significantly negatively correlated with complete valve closure in *L. corrianus* and *P. (P.) gowhattensis* in 1% Cu-exposed group. Extensive extension of foot and siphon together was significantly negatively correlated with complete valve closure in *L. corrianus* in 10% Cu-exposed group. Significantly movement was negatively correlated with valve closure in *L. corrianus* and *P. (P.) gowhattensis* in 10% Cu-exposed group (Suplementary Table 1). No significant correlation was observed between crawling speed and duration of head-foot protrusion in 1% and 10% Cu-exposed groups in *M. hainesiana*.

1%: Cu concentration of 1% OF 96 h LC_{50} value, L. corrianus = 0.0847 mgL^{-1}; P. (P.) gowhattensis = 0.001 mgL^{-1}; 10%: Cu concentration of 10% of 96 h LC_{50} value; L. corrianus = 0.8473 mgL^{-1}; P. (P.) gowhattensis = 0.0102 mgL^{-1};

Mov: number of movements/30 min;

FSi: extensive extensions of foot and siphon together in sec/ 30 min;

Vv: complete closing of valves without any extension of foot and siphon in sec/30 min;

** Correlation is significant at 0.05 level; ** Correlation is significant at 0.01 level;*

a: Cannot be computed because at least one of the variables is constant.

The study on behavioral changes due to toxicity was done manually to observe the preliminary changes that could occur to the bivalves and gastropods due to Cu toxicity. The first and foremost behavioral change indicator was the abrupt closing of valve in bivalves and withdrawal of body inside the shell and closing of operculum in gastropod when the Cu metal was introduced into the medium. The molluscs showed rapid response against the entry of Cu in the medium as a protective behavior. This protective behavior was also observed in Pb toxicity in *L. j. obesa* (small and large) and *P. (P.) corrugata* (Brahma and Gupta 2020). Molluscs are filter feeders and closing of valves and operculum may be a strategy to avoid entry of metals or toxins in the animal body, also have been explained in studies of *I. obsoleta* by Clotfelter et al. (2004) as "early warning". There was no mucus secretion observed during the behavioral tests in Cu exposed media in both bivalves and gastropod, the reason might be due to very low concentrations were used in all the three species of molluscs. Otherwise, in acute toxicity tests of molluscs studied, a milky white mucus substance was found to be secreted by the molluscs. The mucus secretion was also observed in *L. marginalis* in toxicity tests to cadmium (Cd) (Yasmeen *et al.* 2012), lead (Pb) in *L. j. obesa* (small and large) and *P. (P.) corrugata* (Brahma and Gupta 2020).

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