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Toxicity of Cu^{2+}, Cd^{2+} and Pb^{2+} Metal Ions in Chironomids and Factors that Affect Metal Accumulation

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Abstract— **The protection of aquatic habitat from damage requires proper assessment of the degree of ecosystem degradation and understanding of both the sensitivity of aquatic organisms to contaminants and their ecological requirements. Assessing the sensitivity of these organisms to contaminants was conducted through a series of toxicity tests. The 96-hour toxicity of Cu, Cd and Pb in chironomids was investigated. The LC50 obtained were 1.37 µg/mL for copper, 73.09 µg/mL for cadmium, and 38.47 µg/mL for lead. This gave an order of toxicity of Cu>Pb>Cd for chironomids for 96 hours of exposure. The three metals exhibited an increase in accumulation with increasing concentration of test solution. Among the three metals, lead was observed to be the most accumulating since it yielded the highest concentration in organisms. However, from the LC50 obtained, it shows that cadmium is the least toxic. For Cd^{2+} and Pb^{2+} ions, accumulation in organisms decreased with presence of Na^{+} and Ca^{2+} in the test solution. Furthermore, for a mixture comprising of three metal ions, it was also found that there was a reduction in the amount of metals absorbed by the organisms and caused a lower concentration on organisms as compared when exposed to a single metal system which may be attributed to competition.**

Key words-toxicity, accumulation, chironomids (or Chironomus sp.), LC50, metal ions

I. INTRODUCTION

Surface waters often serve as sink for various toxicants of the environment through natural and anthropogenic processes. Heavy metals received major attention because of their persistent toxic effect and their ability to be accumulated within compartments of the environment. They bind to organic and inorganic particles, which in the long run, may also settle at the bottom of streams, rivers, lakes, estuaries or marine waters. This can lead to deleterious effect to benthic organisms, fish, and potentially on human health through ingestion of metal enriched sediment and through uptake from solution [1].

Elevated concentration of metals was clearly observed on streams and rivers which receive wastes from mining processes [2-4]. Metal concentration in the sediment and water declined after a period of time, but concentration among benthic insects remained relatively unchanged [4]. Therefore, the protection of aquatic habitat from damage due to such contaminants requires proper assessment of the degree of ecosystem degradation and understanding of both the sensitivity of aquatic organisms to contaminants and their ecological requirements.

Toxicity refers to capacity of a test material to cause adverse effects on living organisms resulting from exposure concentration and time. In this study, toxicity is measured in terms of median lethal concentration (LC50). The midges (order Diptera, family Chironomidae) were used in evaluating the toxicity of Pb^{2+}, Cd^{2+}, and Cu^{2+} in a controlled condition. These organisms account for most of the macroinvertebrates in freshwater environments. The greatest part of their life cycle, the larval stage, is spent in aquatic environment which made them continuously exposed to contaminants and therefore, can reflect aquatic environment degradation.

Accumulation of metal in organisms could be through intake of contaminated food (including sediment) or through exposure to contaminated water. The incoming metal will thus encounter a wide range of potential binding sites, which usefully divided into two types: physiologically inert sites, where the metal may collect without obviously perturbing all normal function, and physiologically active sites where the metal affect cell metabolism. Storage of metal in inert form is possible through those tissues rich in or capable of synthesizing large quantity of metal binding ligands which include various types of granules and metal binding proteins such as metallothionein. Furthermore, few species have the ability to eliminate metal through excretion [5].

This study enumerates few factors which may tend to influence the transport and bioaccumulation of metals from

the immediate environment to benthic macro invertebrates such as the chironomids. Metal accumulation with mixture of the metals was evaluated. Accumulation of metals can be affected by interaction of metals that resulted to stimulation and antagonism [1]. Metal concentration of each test solution and test organisms that survived was verified after each test using Atomic Absorption Spectrophotometry. All analyses conducted with chironomids for the determination of metal accumulation, involved the whole body of organisms.

II. Materials and Methods

A. Culture of Chironomids

Midge larvae used were collected from an uncontaminated creek in University of the Philippines, Los Baños. These were placed in a glass container, covered with a net to trap emerging adults. Since the aquarium already contains male and female species, mating and production of eggs is possible. To produce eggs of similar age, each egg mass collected was placed on a beaker containing 25 mL of tap water that was aged overnight. After two days, when all eggs were hatched, larvae were transferred on a 14 inches x 10 inches x 6 inches aquarium and given fish flakes for food. This was the source of all test organisms. Shredded tissue paper was used as substrate. Overlying water was being replaced every three days. Table 1 summarizes the conditions for the culture of test organisms.

TABLE I. Conditions for the Culture of Chironomids

Parameter	Specification
pH	7 -8
Temperature	27°C
Dissolved oxygen	7 ppm
Photoperiod	16:8 hours light:dark
Aeration	Low intensity
Dilution water	Tap water
Feeding regime	0.025g fish flakes/day

B. Toxicity Test of Metals

Test solutions used for the acute toxicity test were prepared by dissolving proper amount of $Pb(NO_3)_2$ (Merck, 99.5% purity), $CdCl_2$ (HiMedia Lab, 98% purity), and $CuCl_2$ (Ajax Chemicals, 98% purity) in distilled water producing 1000ppm of stock solution. From these stock solutions, different concentrations of test solutions were prepared through a series of dilution.

An initial range finding test was performed (starting from 50ppm to 1000ppm) to determine the concentration of each metal where test organisms been exposed for 96 hours. The concentrations of each metal are increased or decreased in the next experiment, depending on the rate of mortality obtained in the initial range finding test. This procedure was repeated until the toxic range was covered for each substance.

Tests were static. Third instar organisms were placed individually in micro test tubes filled with test solutions. Each concentration consists of three trials. A control was also prepared wherein organisms were exposed to distilled water. They were not fed during the toxicity test. Shredded tissue paper was added, just enough for the organisms to create their own tubes. Absence of tube where they could dwell causes a mortality of higher than 10% even in the absence of any toxicant. The tube made by chironomids functions in feeding and respiration. After 96 hours, surviving test organisms were determined and the mortality percentage was recorded. The criterion for death is immobility and/or lack of reaction to mechanical stimulus. Mortality percentage was calculated using Abbot's formula.

Median lethal concentration was calculated using Probit analysis. The values of % mortalities are being transformed to probit values. Logarithm of concentration was plotted against these values. The LC50, LC16 and LC84 were extracted from this graph by taking the antilogarithm of the logarithmic concentration corresponding to the appropriate probit values for this percentage. The 95% confidence interval was calculated using the Litchfield-Wilcoxon method. Table 1 summarizes the conditions for the culture of test organisms.

TABLE II. Length of Chironomids at Different Larval Stage

Larval stage	Age, days	Length, mm
1st instar	0 to 3	1.56 ± 0.67
2nd instar	3 to 9	4.78 ± 0.56
3rd instar	10 to 11	7.7 ± 0.86
4th instar	14	10.6 ± 0.66

C. Test for Accumulation of Each Metal

Similar concentrations of test metal solutions that used in the toxicity test were also used for bioaccumulation. Polyethylene cup filled with 125 mL of stock solution with 0.5, 1, 3 and 5ppm of Cu^{2+}, 10, 20, 50 and 100 ppm of Cd^{2+}, and 5, 10, 40 and 100 ppm of Pb^{2+} were used as test solutions. Thirth (30) organisms were placed in each container and exposed for 96 hours. The second batches chironomids were exposed in a solution consisting of 1 ppm Cu^{2+}, 50 ppm Cd^{2+} and 40 ppm Pb^{2+}. The third batch of organisms were exposed to solution containing each metal with the addition of 20 ppm Na^{+} and 50 ppm Ca^{2+}. The organisms that survived were isolated, placed in a beaker and dried at 60°C until a constant mass was achieved. The samples were then soaked in 15 mL of 1:1 HNO_3/H_2O_2(38%v/v) overnight and then digested until the solution turned syrupy. This solution was filtered using whatman #2 filter paper. It was then diluted into 25 mL, and analyzed for the metal content using the AAS (Perkin Elmer AAnalyst 100 Spectrometer). The slit width for all the metals is 0.7 nm. The wavelength for Pb is 283.3 nm, for Cu is

324.8 nm and for Cd is 228.8 nm. The flame type is air/acetylene.

D. Determination of the Total MetalContent of Test Solution

For the determination of heavy metals, 100 mL of acidified water sample obtained from the test solutions was initially digested. After digestion, the sample was filtered and transferred to a 100 mL volumetric flask and diluted to mark with distilled water. Standard solutions of Cu^{2+}, Cd^{2+}, and Pb^{2+} were prepared. From the stock solution, a joint stock solution containing 0.05, 0.1, 0.2, 1.0, 5.0, 10.0 and 25.0 ppm of Cu^{2+}, Cd^{2+}, and Pb^{2+} were prepared. The concentration of metals in the sample was determined from a plot of absorbance readings of standards.

III. Results and Discussion

A. Toxicity of Cu^{2+}, Cd^{2+} and Pb^{2+}

The 96-hour toxicity of Cu, Cd and Pb in chironomids was investigated. The LC50 obtained were 1.37 µg/mL for copper, 73.09 µg/mL for cadmium, and 38.47 µg/mL for lead. This gave an order of toxicity of Cu>Pb>Cd for chironomids for 96 hours of exposure. The three metals exhibited an increase in accumulation with increasing concentration of test solution. Among the three metals, lead was observed to be the most accumulating since it yielded the highest concentration in organisms. However, from the LC50 btained, it shows that cadmium is the least toxic.

TABLE III. LC50, 95% Confidence Limit and Other Variables

	Cu	Cd	Pb
LC 16	0.35	27.19	6.65
LC 50	1.37	73.09	38.47
LC 84	5.37	196	222
S*	3.926	2.685	5.779
N'**	87	90	118
f_{LC50}***	1.501	1.334	1.560
Upper 95% CI	2.05	104	60.01
Lower 95% CI	0.91	54.79	24.66

*slope
**total number of individuals tested between the 16% and 84% response
***S (raised to 2.77 ÷ square root of N')

From the concentration-response obtained, it could be seen that copper is the most toxic among the three metals. Concentration higher than 1 ppm was already fatal. Its easy transport through cell may have contributed to the immediate effect of copper to some of their physiological functions. Toxic effect of cadmium on the other hand, results from a reduced uptake of essential metals. Cadmium, accumulates pathway of essential metals specifically Ca^{2+} channel due to their similarity of size and charge. Entry of Cd^{2+} through Ca^{2+} conducting ion channels provides Cd^{2+} with direct access to any pathway in which it can substitute for or disrupt the normal physiologic actions of Ca^{2+}. Lead metal ion likewise, has this kind of ability to inhibit or mimic the actions of Ca^{2+} which can affect calcium-dependent or related processes [6].

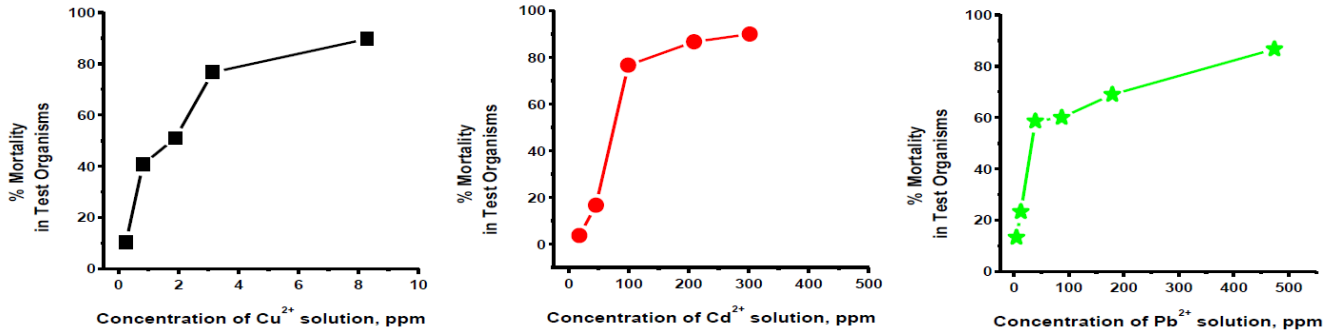

Figure 1. Concentration vs % Mortality in Chironomids.

Between Cd^{2+} and Pb^{2+} in this study, chironomids are more tolerant towards Cd^{2+}. From previous literatures, it is stated that *chironomus sp* contains metallothionein (MT), a metal binding protein (MBP) [7]. MT was originally discovered as a cadmium binding protein. Cellular tolerance to Cd^{2+} is thought to occur through high affinity sequestration of the toxic metal by MT. Metallothionein contains numerous thiol groups due to their very high cysteine content which provides the basis for high cysteine content which provides the basis for high affinity binding of metals.

Aside from being stored in insoluble inclusions among organisms and being bonded with metal-binding ligands such as MT, the organisms could also have been protected by their self-made tubes. In 2002, Halpern et al.[8], found in his study that self-made tubes which function for feeding and respiration gives also protection to the organisms. The tube

serves as site for larva's own protein secretion. This protein plays a role in protecting the larva against toxicants.

B. *Factors that Affect Accumulation Cu^{2+}, Cd^{2+} and Pb^{2+} in Chironomids*

The three metals exhibited an increase in accumulation with increasing concentration of test solution (Figure 2). From 0.48 to 0.98 ppm of Cu^{2+} metal solution, the concentration of Cu in organisms rises. At concentration higher than 1 ppm, copper solution exhibited 50% mortality among organisms. In the case of lead, concentration in organisms rises from 4.9 to 12.2 ppm of Pb^{2+} solution. Chironomids that accumulated Pb could still afford to survive but not at near 40 ppm because at this concentration, the mortality obtained was 50%. The rest of the organisms that survived might have exhibited different tolerance mechanisms.

Also, it could be noticed that in the case of chironomids exposed in lead solution, metal uptake in organisms reaches about 19,000 ppm. This concentration of Pb in organisms is extremely high. Previous study of Grosell 2006 [9] mentioned that presence of CO_2 in water produces HCO_3^- in aqueous solution that could result to $PbCO_3$ precipitation. This precipitate could have settled and have directly ingested by organisms but have stored in inert form. Previous literature obtained residual fractions of Pb in insects that could be comprised of metals absorbed to external body parts or insoluble, intracellular metal [10]. Accumulation of Pb among chironomids may have occurred through adsorption or ingestion but converted to insoluble fractions. Only those portion of Pb that have transported intracellularly, contributed to toxic effects of lead.

For Cd^{2+} ion, uptake of Cd by chironomids is proportional to the concentration of solution. Tolerance of test organisms with high concentration of Cd^{2+} may suggest that *chironomus sp* used in this experiment possesses MT like other species of chironomids used in previous literatures [7,11] which reduces the toxicological capability of cadmium.

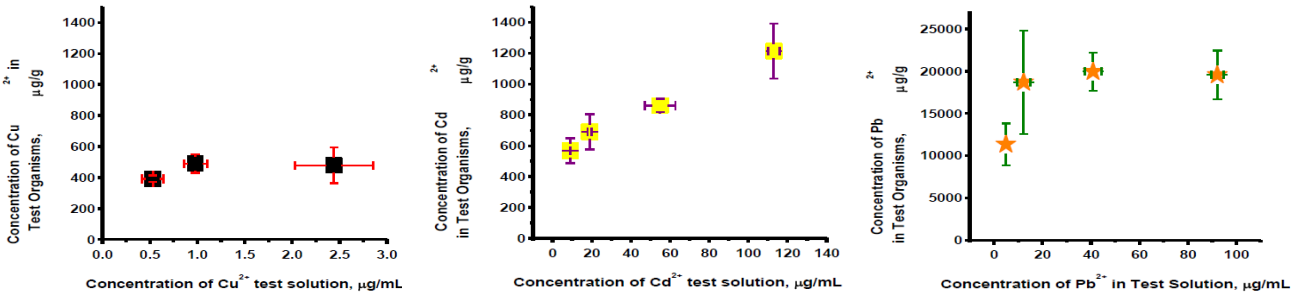

Figure 2. Metal Content in Chironomus sp. Vs Metal Content of Test Solution.

Chironomids were exposed to a mixture of metals with concentrations of 1.34 ppm of Cu^{2+}, 56.56 ppm of Cd^{2+} and 38.41 ppm of Pb^{2+}. Accumulation of metals by chironomids relatively decreased when they were exposed to mixture of the three metals as compared when exposed to individual metal of similar concentration (Figure 3 a-c). This may be due to the competition of the three metals for binding sites on the cell surface of chironomids. Antagonism among available metals could decrease accumulation to organisms.

Aside from the mixture of the three metals, the organisms were also exposed to each metal in the presence of Ca^{2+} and Na^+. Results showed that Cu accumulation was not affected by the presence of Na^+ and Ca^{2+} metal ions. Copper toxicity to several aquatic species has been reported to be negatively correlated with hardness, but other reports have indicated little or no effect [12,13]. In this study, neither Ca^{2+} nor Na^+ could interfere in the accumulation of copper among chironomids. This is despite of the increase of Na^+ and Ca^{2+} in the organism due to the mixing of these metals in the test solution. However, Cd^{2+} and Pb^{2+} accumulation decreased in the presence of Ca^{2+} and Na^+. Previous study showed that Ca^{2+} interfered with the uptake of Cd^{2+} metal ion either by competing in transport through cell membranes or by reducing membrane permeability [6]. Since Na^+ and Ca^{2+} are essential ions, they have specific route that made them competitive in transport to the organisms. Metal toxicity is also related to disruption of ion exchange and that mortality is correlated to drop of Na^+ levels. External Na^+ could assist during disruption of ion exchange caused by metal influx. Consequently, accumulation and toxicity of metals have decreased.

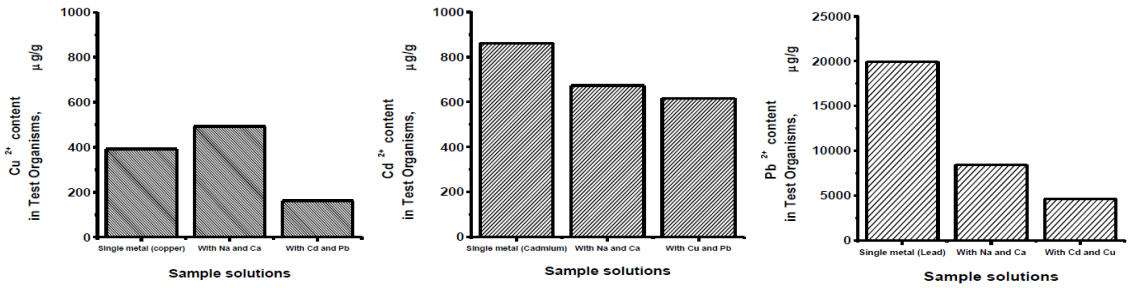

Figure 3. Metal Content in *Chironomus sp* in Single and Multiple Metal System.

The high tolerance of chironomids to Pb^{2+} and Cd^{2+} could also be understood if specific metabolism of each metal will be established. Previous literature found that Pb is being transported to insoluble granules while Cd is bound to metal-binding ligands [10]. Accumulation in chironomids can be understood by examining subcellular partitioning of accumulated metal described by Wallace *et al*., 2003[14]. Compartmentalization approach could be done to interpret the significance of metal, species, and size dependence in the subcellular partitioning of metals in the organisms. Metals could be compartmentalized as metal-sensitive fractions (MSF) (i.e. organelles and heat-sensitive proteins) and biologically detoxified metal (BDM) (i.e. metallothinein [MT] and metal-rich granules [MRG]). Target sites of each metal could explain its toxicity. Detoxification mechanism of these organisms could also explain their high tolerance towards Pb and Cd. An example was discussed by Timmerman and Walker 1989 [5] about elimination pathways of trace metals during metamorphosis of chironomids.

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