

Chironomids as Biological Indicators of Metal Contamination in Aquatic Environment

Claire C. Lagrana, Dahlia C. Apodaca, and Carlos Primo C. David

Abstract— Chironomids belong to the group of benthic insects that could reflect aquatic environment degradation. A 96-hour toxicity test for Cu, Cd and Pb in chironomids was investigated and the LC50 obtained were 1.37 µg/mL for copper, 73.09 µg/mL for cadmium, and 38.47 µg/mL for lead. The three metals exhibited an increase in accumulation with increasing concentration of test solution. Among the three metals, Pb yielded the highest concentration in organisms. The result suggests that some Pb may have accumulated in physiologically inert sites in the body of organisms. However, from the LC50 obtained, it shows that cadmium is the least toxic. The organisms used in this study might have the ability to synthesize metallothionein, a metal-binding protein which could reduce the toxicological capability of Cd. A multiple-metal system was also employed to monitor metal accumulation in chironomids. It was found that there was a reduction in the amount of metals absorbed by the organisms when compared to organisms exposed to a single metal system.

Index Terms—Accumulation, biological indicator, chironomids (or *Chironomus* sp.), median lethal concentration, metals

I. INTRODUCTION

Freshwater habitats act as a sink for naturally occurring wastes as well as for different anthropogenic toxic substances. 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].

Mining processes have resulted in extensive trace metal contamination in streams and rivers by the downstream transport of waste material [2-4]. A bioaccumulation study conducted in a tropical asian stream (the Philippines) showed that 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.

Benthos is the community of organisms which live near the seabed, also known as the benthic zone. Because light does not penetrate in very deep ocean-water, the energy source for deep benthic ecosystems is often organic matter where contaminants are bound. The midges (order Diptera, family Chironomidae) account for most of the macro invertebrates 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, a good biological indicator of 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 was conducted to complement the results of field studies employing benthic insects as indicator of metal contamination. Chironomids were used as test organisms. The median lethal concentration (LC50) of Cu, Cd and Pb in chironomids using 96-hour of exposure time was determined. This reflects the concentration which causes 50% mortality among the test organisms. This study also 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. A multiple-metal system, a reflection of a genuine river, was also employed to monitor metal accumulation. Accumulation of metals can be affected by interaction of metals that resulted to stimulation and antagonism [1]. 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

The larvae used were collected from an uncontaminated creek I in University of the Philippines, Los Baños. These were placed in a glass container, covered with a net to trap

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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 $\text{Pb}(\text{NO}_3)_2$ (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 percentage 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 II. LENGTH OF CHIRONOMIDS AT DIFFERENT LARVAL STAGE

Larval stage	Age, days	Length, mm
1 st instar	0 to 3	1.56 ± 0.67
2 nd instar	3 to 9	4.78 ± 0.56
3 rd instar	10 to 11	7.7 ± 0.86
4 th 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 batch 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 $\text{HNO}_3/\text{H}_2\text{O}_2$ (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 Metal Content 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+}

Results showed the direct relationship between concentration of metals and mortality of organisms (Fig. 1). The LC50 obtained using 96-hour of exposure time in chironomids 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 $\text{Cu} > \text{Pb} > \text{Cd}$. Table III shows the confidence limit and other variables calculated. The three metals exhibited an increase in accumulation with increasing concentration of test solution. This complements the results of field studies employing benthic insects as indicator of metal contamination [2-4]. Among the three metals, lead yielded the highest concentration in organisms (Fig. 2). However,

from the LC50 obtained, it shows that cadmium is the least toxic.

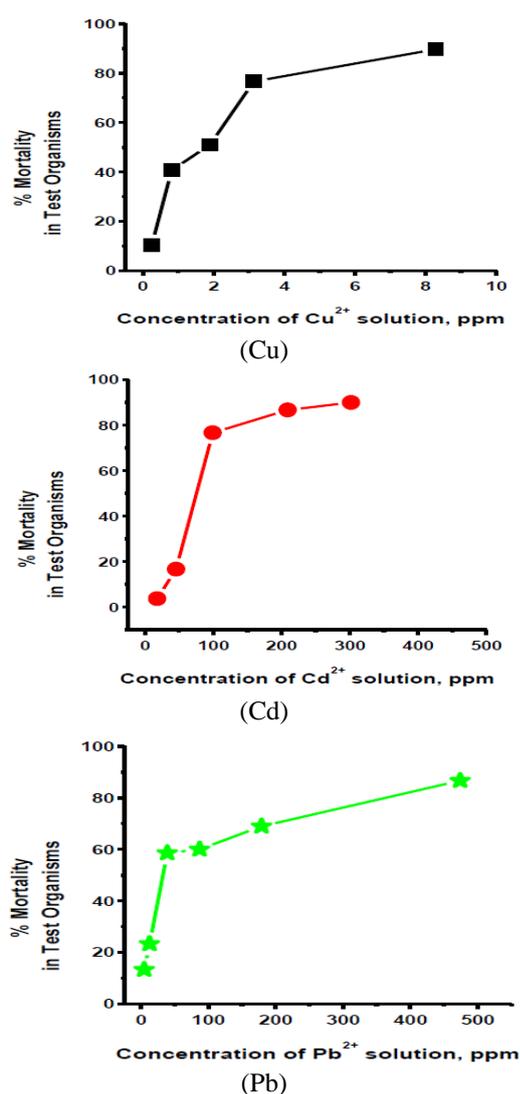


Fig. 1. Concentration vs % Mortality in Chironomids.

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 $2.77 \div$ 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+}

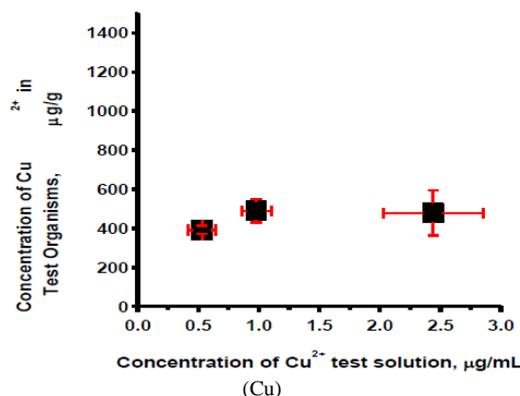
channel due to their similarity of size and charge. Entry of Cd^{2+} through Ca^{2+} conducting ion channels provides Cd^{2+} with direct access to any pathway in which it can substitute for or disrupt the normal physiologic actions of Ca^{2+} . Lead metal ion likewise, has this kind of ability to inhibit or mimic the actions of Ca^{2+} which can affect calcium-dependent or related processes [6].

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* can synthesize 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 affinity binding of metals. High tolerance of chironomids to Cd suggests that the species employed in this study possibly synthesized metallothionein which caused the reduction of toxicological capability of Cd.

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 (Fig. 2). This complements the results of field studies employing benthic insects as indicator of metal contamination. 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. For Cd^{2+} ion, uptake of chironomids reaches 800 ppm in the region where 50% of the population exhibited mortality. However, there are number of organisms that could still accumulate more of the Cd which may be attributed to the synthesis of MT.



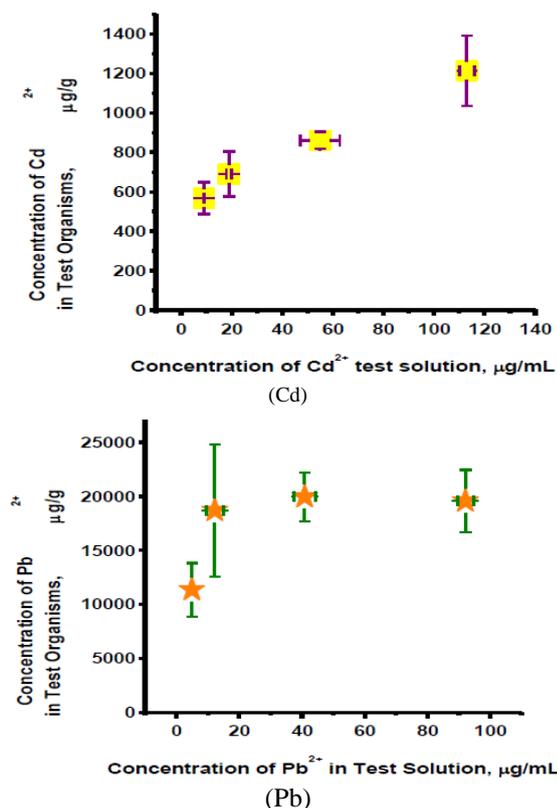


Fig. 2. Metal Content in *Chironomus* sp. Vs Metal Content of Test Solution.

Among the three metals, lead yielded the highest concentration in organisms. This concentration of Pb in organisms is extremely high. Previous study of Grosell 2006 [9] mentioned that presence of CO₂ in water produces HCO₃⁻ in aqueous solution that could result to PbCO₃ 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.

Chironomids were exposed to a mixture of metals with concentrations of 1.34 ppm of Cu²⁺, 56.56 ppm of Cd²⁺ and 38.41 ppm of Pb²⁺. 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 (Fig. 3). 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.

A multiple-metal system, a reflection of a real river, was also employed to monitor metal accumulation in chironomids. The organisms were also exposed to each metal in the presence of Ca²⁺ and Na⁺. Results showed that Cu accumulation was not affected by the presence of Na⁺ and Ca²⁺ 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 Ca²⁺ nor Na⁺ could interfere in the accumulation of copper among chironomids. This is

despite of the increase of Na⁺ and Ca²⁺ in the organism due to the mixing of these metals in the test solution. However, Cd²⁺ and Pb²⁺ accumulation decreased in the presence of Ca²⁺ and Na⁺. Previous study showed that Ca²⁺ interfered with the uptake of Cd²⁺ metal ion either by competing in transport through cell membranes or by reducing membrane permeability [6]. Since Na⁺ and Ca²⁺ 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 of metals have decreased.

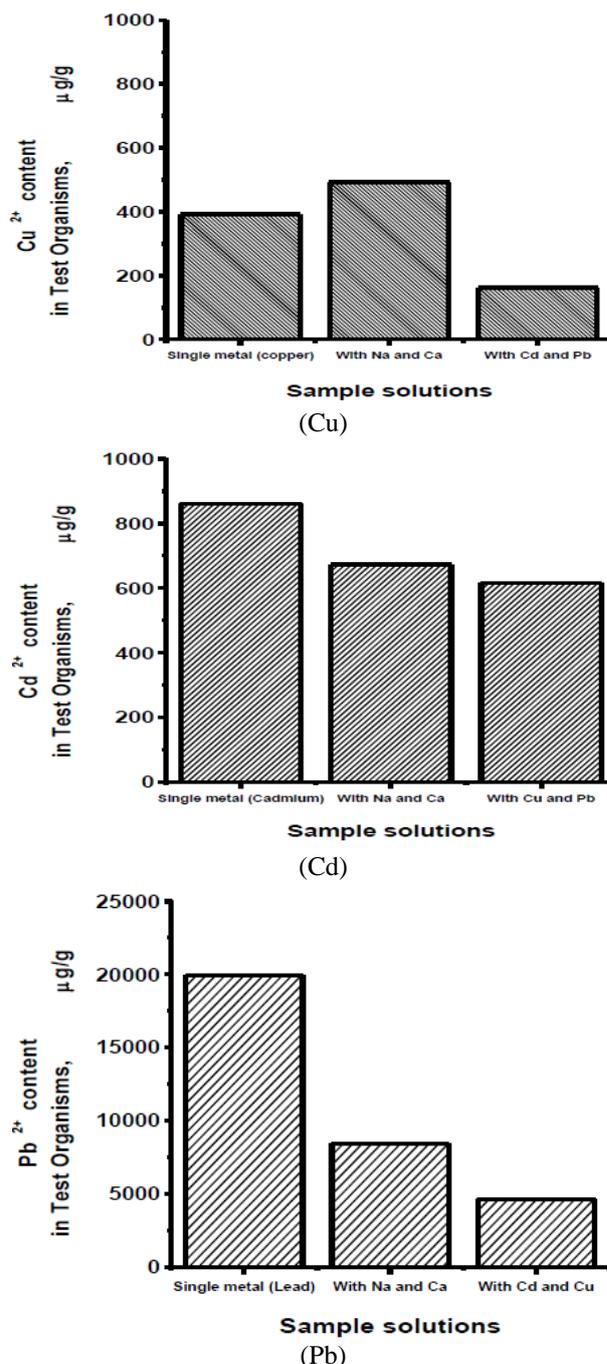


Fig. 3. Metal Content in *Chironomus* sp. in Single and Multiple Metal System.

Results obtained in this study proved that chironomids can accumulate excessive amount of trace elements. These

organisms therefore can be a good indicator of aquatic environment degradation. The high tolerance of chironomids to Pb^{2+} and Cd^{2+} could 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. metallothionein [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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