Genotoxicity of Two Heavy Metals, Pb and Cd on *Perionyx excavatus*, an Epigeic Earthworm.

Prasanta Mandal, Rupa Dasgupta, Jayanta Kumar Kundu

Abstract—There is a growing public concern over the potential impact of accumulation of heavy metals in soil. In the present study, 96 h LC$_{50}$ value of two heavy metals – lead (Pb) and cadmium (Cd) were evaluated on *P. excavatus*, an indigenous epigeic earthworms under laboratory conditions as per OECD guidelines 1984(207) and the effect of sub-lethal doses of the metals on the coelomocytes was evaluated using micronucleus test. The total mortality of the test specimens observed after 96 hours of exposure of the Pb and Cd applied in the form of lead nitrate [Pb(NO$_{3}$)$_{2}$] and cadmium nitrate [Cd(NO$_{3}$)$_{2}$] to a range of doses were subjected to probit analysis to determine LC$_{50}$ value at 95 % confidence limit for each heavy metal. In sub-lethal toxicity studies (96h), earthworms were exposed to a range of Cd and Pb concentrations corresponding to 30%, 50% and 70% of LC$_{50}$ values of each metal on *P. excavatus*. The LC$_{50}$ values on *P. excavatus* were evaluated to be 2.975 gkg$^{-1}$ and 1.418 gkg$^{-1}$ for Pb and Cd respectively. In sub-lethal tests, with exposure to Cd at 0.709 gkg$^{-1}$ (50% 96-h LC$_{50}$), significant increase in micronuclei of coelomocytes of test organisms were noted indicating possible chromosomal breakage and aberrations. There was also a significant increase in binucleated coelomocytes for Cd compared to Pb at each concentration indicating that Cd caused greater failure in cytokinesis in the form of binucleated cells. Again, micronuclei frequency of the coelomocytes even up to exposure concentration of 1.488 gkg$^{-1}$ of lead were not affected indicating its comparative less toxic nature. Evaluated results indicate that Cd may be categorized as comparatively “more toxic” and Pb can be classified as “moderately toxic” to the test organisms.

Index Terms—Lead nitrate, Cadmium nitrate, Earthworms, Acute toxicity; 96 h LC$_{50}$, Micronucleus test.

1 INTRODUCTION

Metals occur naturally in soils as a result of diverse geological processes such as chemical reaction and erosion of underground geological materials [1]. Beside these natural sources, industrial activities can supply a considerable quantity of metals to soil [2]. A large number of industrial activities produce wastes and contaminants that reach the soil through direct disposal, spills, leaks, atmospheric deposition from air, and other pathways [3]. However, the highest mean Cd concentrations were found from the textile industry, while the highest Pb from cement plant, battery production industry and scrap battery recovery facilities. In addition to the sources mentioned above, thermal power plants, and iron-steel industries are commonly found to be major industrial sources of Pb [4].

Edaphic organisms can be used as a first screening tool for soil Environmental Risk Assessment. Generally, mortality is not a very sensitive endpoint for predicting the effects of a toxicant on field populations. It is assumed that measures like LC$_{50}$ are crude indices of biological effects.

In polluted soil, earthworms are exposed to these pollutants both dermally and through gastrointestinal tract absorption from soil. Inorganic Pb is classified as a group IIA carcinogen, which is a probable human carcinogen [5]. Cadmium is classified as a group I human carcinogen [6] and an animal carcinogen [7].

The present study aims to determine the toxic effects of lead and cadmium concentrations, administered in the form of lead nitrate and cadmium nitrate, on *Perionyx excavatus*, an indigenous, epigeic earthworm widely recognized as a model test organism for ecotoxicological risk assessment of polluted soil. Acute Toxicity studies were conducted to determine Median lethal concentrations of lead and cadmium on *P. excavatus* and the micronucleus test has been used as a cytogenetic technique to detect chromosomal aberration and nuclear abnormalities in the form of binuclei and micronuclei of the test organism with exposure to sub-lethal concentrations of the two heavy metals.

2 Materials and Methods

2.1 Test Organisms

Earthworms are more susceptible to metal pollution than many other groups of soil invertebrates, and toxicity data on earthworms are important in determining “safe levels” for metals and other contaminants in soil [8]. Keeping in context of the use of earthworms as model test organisms for ecotoxicological risk assessment of soil, the present study was carried out using *P. excavatus*, an indigenous, epigeic earthworm as the test organism. Specimens of *P. excavatus* were collected from uncontaminated grassland around Midnapore town (West Bengal, India). Age synchronized clitellate earthworms with body weight between 250-300 mg were used for the experiments.
2.2 Test Chemicals
Cadmium nitrate [Cd(NO\(_3\)]\(_2\)\] and lead nitrate [Pb(NO\(_3\)]\(_2\)\] were used as test chemicals. These chemicals were purchased from the scientific chemical shop, Purba Medinipur, West Bengal, India. At first, range-finding tests were performed to determine the concentrations to be used in the tests. The range of Cd and Pb concentrations were chosen to mimic the levels of these metal in typical non-contaminated and in industrial contaminated soil.
Concentrations to be used were determined to be 2, 4, 6, 8 gkg\(^{-1}\) and 1.5, 2.0, 2.5, 3.0 gkg\(^{-1}\) for Pb and Cd respectively in dry soil. A calculated mass of lead nitrate and cadmium nitrate were weighed for each desired concentration and each was dissolved in 60 ml distilled water. The solutions of lead nitrate and cadmium nitrate were sprayed on the soil (200 g dry mass) to get the desired concentrations.

2.3 Acute Toxicity Studies
Bioassays were made with age synchronized specimens in small inert polythene boxes (16x12x1cm; total area, 192 cm\(^2\) each containing 200 g of a test medium. The samples of the test medium were dried, ground and sieved to get a particle size of 0.25 mm before lying in the experimental boxes. Soil moisture of the test medium was determined and kept constant throughout the experiment. Different doses of heavy metals (gkg\(^{-1}\)) calculated to mimic the actual concentrations in natural polluted soil were administered into the test boxes with a micropipette. The experiment was set up with three replicates for each dose of the heavy metal and control. The boxes were then left undisturbed for about 30 minutes for uniform spreading of the heavy metal in the medium. Fifteen numbers of age synchronized test specimens were then transferred into the boxes. Finally the experimental boxes were kept in a BOD incubator at a constant temperature of 28 ± 0.5°C and 50 - 60% moisture [9]. Observations were made every 24 hours. Those individuals, who showed no apparent sign of life, even when poked with a needle, were considered dead and were removed. Mortality of the test specimens after 24, 48, 72 and 96 h of observations were recorded.

2.4 Sub-lethal Toxicity Studies
In sub-lethal toxicity studies, earthworms (n = 10 per concentration) were exposed to sub-letal concentrations of Cd and Pb concentrations up to 96-h, 0.893, 1.488, 2.083 gkg\(^{-1}\) soil and 0.425, 0.709, 0.993 gkg\(^{-1}\) soil for Pb and Cd respectively. No chemical was used in the control. After 96-h, worms from each concentration were removed from the soil, washed in distilled water, and blotted on a paper towel.

2.5 Harvesting Coelomocytes
Some quantities of earthworms (±15gms) were subjected to cold shock by using ice cubes in a petriplates and the fluid was collected in a clean dry test tube [10]. Through this method, about 1.5 ml of coelomic fluid was collected in 30 minutes.

2.6 Micronucleus Test
2.6.1 Slide preparation
Aliquots of 20 μL of coelomic fluid were smeared on clean microscopic slides using one slide for each replicate (a total of three slides per concentration). After the slides were air-dried, the cells were fixed in absolute ethanol for 15 min, stained with 5% Giemsa solution for 8 min and washed under the tap water [11].

2.6.2 Calculation
The mitotic stage was examined with a binocular microscope. A total of 3,000 small coelomocytes or body cell from three separate slides (1,000 cells per slide) per concentration were scored using a compound microscope(Magnus MLX PLUS Photo Microscope) at 1,000x magnification to determine the micronucleate (MN) and binucleate(BN) frequencies. The frequencies of MN and BN cells were evaluated as the number of abnormalities per 1000 cells (%) scored [12].

2.7 Statistical Analysis
The total mortality of the test specimens obtained after 96 hours of exposure were subjected to probit analysis by EPA probit analysis program, version 1.5 (US EPA, 2006) to determine LC\(_{50}\) value and 95 % confidence limit of each heavy metal. In the sub-lethal study, results were expressed as the mean from three replicate. To compare the results of micronucleus study, “t” test was done in SPSS (version 20).

3 Results
3.1 Acute Toxicity Test
Earthworms exposed to various concentrations of lead nitrate and cadmium nitrate showed marked different toxicities after 96h of exposr in terms of mortality differing from the control. The LC\(_{50}\) was thus evaluated as 2.975gkg\(^{-1}\) for Pb and 1.471 gkg\(^{-1}\) for Cd in P. excavatus. Thus, Cd is comparatively more toxic than Pb to the test organism (Table 1).

Table 1: 96 h LC\(_{50}\) values of Pb & Cd for P. excavatus.

<table>
<thead>
<tr>
<th>Heavy Metal</th>
<th>96h LC(_{50})</th>
<th>95 % Confidence limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pb</td>
<td>2.975 gkg(^{-1})</td>
<td>1.574 - 4.005</td>
</tr>
<tr>
<td>Cd</td>
<td>1.418 gkg(^{-1})</td>
<td>0.724 - 2.61</td>
</tr>
</tbody>
</table>

3.2 Micronucleus Test
was higher than that observed with Pb (6.33±1.20 and 11.67±0.88) (Fig.1, 2)

4 CONCLUSION
The LC₅₀ of values determined on P. excavatus were evaluated to be 2.975gkg⁻¹ and 1.418 gkg⁻¹ for Pb and Cd respectively. The biomarker potential of mortality in P. excavatus were evident within 96 hours of exposure to Pb and Cd. Evaluated results showed that Cd is comparatively “more toxic” and Pb can be classified as “moderately toxic” to the test organism. Greater toxicity of Cd compared with Pb may be due to the differences in bioavailability and/or absorption and/or compartmentalization of the two metals in the earthworms. This is supported by the studies of Nahmani et al., 2007[13] and Ma, 1982[14].

In the sub-lethal 96-h study, both Cd and Pb increased the binucleate coelomocytes. Binucleate cells occur due to a defect in cytokinesis. Our result demonstrated that Cd concentration (0.425 gkg⁻¹) required to induce binucleate coelomocytes was approximately 50% of that required for Pb(0.893 gkg⁻¹). This finding agrees well with the study of Conder and Lanno, 2002[15] who reported that Pb is only slightly toxic and relatively well tolerated by the coelomocytes, compared to Cd, due to sequestration of Pb by chloragogen cells. Morgan and Morgan, 1998[16] also reported that earthworms store lead in a non-toxic form and die when the stored Pb reaches a critical concentrations. This is supported by the fact that exposure to lead did not affect micronuclei frequency even up to exposure concentration of 0.893 gkg⁻¹ in the 96-h sub-lethal study.

In sub-lethal tests, the frequency (mean ± SE) of binucleated coelomocytes at each concentration for Cd and Pb was higher than for micronucleated coelomocytes, compared with the controls. There was also an significant (p < 0.05) increase in binucleated coelomocytes at and above Cd concentration of 0.425 gkg⁻¹(30% of the 96-h LC₅₀) compared with the controls(Fig. 2) . Thus, Cd caused cytokinesis failure in the form of binucleated cells. In contrast to Cd, Pb had no effect on micronuclei formation even at 30% of the 96-h LC₅₀ values (0.893gkg⁻¹)(Fig. 1). The Pb and Cd induced micronuclei frequency were very much closer at 30% of 96-h LC₅₀ (Pb-5.67±1.20 and Cd-6.67±1.20) per 1,000 coelomocytes. But at and above 50% of 96-h LC₅₀ value, the Cd-induced micronuclei frequency (9.67±1.20 and 15.67±1.45) per 1,000 coelomocytes

REFERENCES


