Ecotoxicological Tests of Metal-Contaminated soils

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Abstract. The raw steel production plays a key role in the economic development of Slovakia. Unfortunately, the technology of steel production, agricultural and industrial activities contribute to contamination of the soil in the region Eastern Slovakia. The purpose of this study was to investigate the effect of heavy metals in urban soils from the Košice area, using bioassays on earthworms and phytotoxicity. An earthworm avoidance test has potential advantages for use in evaluation of hazardous soils sites and proved as a quick approach to determining the presence of contaminants. The earthworm (Dendrobaena veneta) takes up and retains metals from soil containing of heavy metals. Assessment of soil phytotoxicity was based on germination and seedling growth of the terrestrial plant (mustard Sinapis alba). The level of Cr, Co, Cd and Hg were assessed in 8 industrial soils (4 agricultural areas and 4 grass-plot areas) from the area U.S.Steel Košice. The highest levels contaminations of chromium (278mg/kg), cobalt (39mg/kg), cadmium (21mg/kg) and mercury (0.80mg/kg) were determined for the grass-plot soils (main gate of the U. S. Steel-plant). Phytotoxicity results for the agricultural soils from around Košice showed that, the potential toxicity values are lowly, represented by a lower percentage of inhibition in germination range between 5 and 33 % and the average percentage of growth inhibition was 12-39 % for Sinapis alba. The results for the grass-plot areas soils from around Košice showed the percentage of inhibition in germination range between 13 and 47 % and the average percentage of growth inhibition was 19-49 % for Sinapis alba. Low mortality effects were recorded in the tests with Dendrobaena veneta. The distribution of the worms found in the double control was within the range 10–100 % for all areas of the agricultural soils and grass-plot soils, for after 48h. The significant (P<0.05) avoidance by Dendrobaena veneta were 100% in soils of areas Gomboš and from main gate of the U. S. Steel-plant, Košice. The above results reflect that not only may the worms be able to detect metals, but in fact they may also change their behavioural response over time.

1. Introduction

In urban regions, land can be contaminated by scattering of metallic elements that develop possible risk to ecosystems. Surfaces of soil are the main sinks for many pollutants, including heavy metals, are prevalent and persistent in the environment, and can accumulate in organisms. The heavy metals may have negative effects on the organisms: they can effect the physical changes (suffocation, preventing access of light), they can change the parameters of the place where organisms live (change of pH, reduction of oxygen concentration, and deterioration of the availability of food) and they are toxic [1, 2]. The aim of this work was to determine terrestrial ecotoxicity of selected heavy metals (Cr, Co, Cd and Hg) in the urban soils from the Košice area, using bioassays (earthworm avoidance tests and phytotoxicity tests). In industrialized areas urban soils are intensively managed.
Košice, the city in eastern Slovakia, is exposed to typical urban contamination sources such as municipal sphere, road traffic, and various industrial sources. Furthermore, being the largest steel producer in Central Europe, it is long-term environmentally loaded by the iron and steel works that represent the largest source of (heavy metals) contamination in Slovakia [3, 4]. The earthworms are continuously exposed to soil chemicals through their surface and skin and dependent on the efficient defoliation systems. Thus, invertebrates (earthworms) are considered suitable organisms to assess the ecotoxicological adverse effects of soil pollution [5, 6, 7]. Avoidance behaviour offers a highly relevant information as it reveals the ability to avoid (or not) possible toxic compounds in the field, hence it provides information on reasons for the presence/absence in the field [8]. Due to their short-exposure periods and their relatively high sensitivity, microbiotests have been successfully used as adequate tools for screening and ranking of the hazards of chemicals and environmental wastes, hence they contribute to a reliable risk assessment for soil environments [9]. Phytotoxkit is an alternative test procedure that enables determination of the biological effects of chemical compounds on plants. Heavy metals are reported to cause delay in germination, reduce the germination percent, inhibition root elongation, seedling development and also affect the seed morphology and physiology. It is confirmed that heavy metals when applied in high doses, decrease seed germination rates as well as root and shoot growth [10, 11]. Nowadays, phytotoxicity tests are receiving increasing attention also in assessing soil toxicity [12].

2. Material and methods
In the year 2017, 8 soils were collected from the Košice suburban, in surrounding of U. S. Steel Košice, which is located about 10km south-westwards from the centre of Košice in Eastern Slovakia. The level of Cr, Co, Cd and Hg were assessed in 8 industrial soils (4 agricultural areas: 1AA-U.S.S. slag heap, 2AA-Gomboš, 3AA-U.S.S. plant west, 4AA-Pereš south, and 4 grass-plot areas: 5GA-U.S.S. slag heap, 6GA-Gomboš, 7GA-U.S.S. plant main gate, 8GA-Pereš south) from the area U.S. Steel Košice.

The soils were sampled at a depth of 20–40 cm into plastic bags. Soil samples were homogenized dried at room temperature; sieved through 2-mm sieve opening. For soil analyses were used control reference soil (CS - MicroBioTests, Belgium). All physico-chemical parameters of industrial soils from areas Košice was described in our previous works [3, 13]. Total concentrations of heavy metals (Cr, Co, Cd and Hg) were determined by the X-ray fluorescence spectrometry method (SPECRO XEPO3). The results were based on soil dry weight.

2.1. Phytotoxicity tests
Evaluation of soil phytotoxicity of the terrestrial plant (mustard Sinapis alba) was using the Phytotoxkit microbiotest (OECD 208). The used phytotoxicity assay and procedure for calculating the percent inhibition of plant (ISG) and/or root growth (IRG) of soils was described in our previous works [11, 14, 15].

2.2. Avoidance behaviour tests
The avoidance tests with earthworms were based on ISO guideline 17512-1/2008 [16]. These tests were designed to separate the effects of the contaminant from those attributable to the physical and chemical properties of soil itself (pH, organic matter and other soil-related factors) [17].

The avoidance test with the Dendrobaena veneta was used to assessing the environmental risk of heavy metals in industrial soils from the Košice suburban. Adult earthworms were purchased from a local supplier, and were allowed to acclimatize for one week in the experimental conditions. The advantage of these organisms is their easy breeding. As control of avoidance behaviour tests, earthworms feed in non-contaminated soil were used CRM. Each replicate consisted of a plastic box (23cm x 13cm x 6cm) divided in two sections. Each section was filled with 250g dry weight of one of the test soils (1-4AA and 5-8GA) and a control soil (CS) on the opposite. Afterward, the divider was
removed, and 10 adult earthworms were placed onto the middle line. The test containers were incubated at 20±2°C and a 16:8 h light: dark photoperiod for 48 h.

At the end of the test period, the test vessels were divided again, and the earthworms in each side of the replicates were counted. Organisms found on the underside of the lid at the end of the test were counted as dead. The percentage avoidance of worms to soils were calculated by counting the mean number of earthworms and compared with the mean number of worms in the untreated control soil. The amount of earthworms counted was converted to a percentage of avoidance. Thus, positive values account for avoidance of worms in test soil, while neutral or negative responses represent indifference or preference of the test substance [11, 18].

2.3. Statistical analysis

In the present study, all experiments were conducted in triplicate and data were expressed as arithmetic mean, median, standard deviations. All data analysed using StatSoft, v.12.0 statistical software [19, 20]. The certified-reference soil GSS1 was used to validation of data.

Data from the phytotoxicity tests and avoidance behaviour response tests were analyzed using the Pearson's matrix correlation. The Pearson correlation coefficients were calculated to study the potential relationships among data of the phytotoxicity tests and concentration of heavy metals. Similarly, data from the avoidance behaviour tests and concentration of heavy metals were analyzed using the correlation matrix.

3. Results and discussions

The descriptive statistical analysis of industrial soils concentrations of heavy metals (means, minimums, maximums, medians, standard deviations) are given in Table 1. The obtained data shows the wide ranges of the concentration of metals. The mean concentration of heavy metals in agricultural soils (AA) was in order of Cd> Co> Cr> Hg. The mean values of the Cr with a mean 156.3mg/kg and median 157.0mg/kg, Co with a mean 54.9mg/kg and median 55.0mg/kg, and Cd with a mean 1.5mg/kg and median 1.8mg/kg, in the area: 2AA-Gomboš was significant higher comparing to the limits the quality of the soil [21]. The authors [2] stated that the concentrations of Cd, Co and Cr in urban soils may originate from manufacturing wastes from industrial factories.

Similarly, mean values of heavy metals in the grass-plot soils were in the order of Cd> Co> Cr> Hg, respectively. In the 7GA-U.S.S. plant main gate soils showed high contamination values for the Cr with a mean 274.7mg/kg and median 273.5mg/kg, Co with a mean 38.2mg/kg and median 38.5mg/kg, Cd with a mean 21.3mg/kg and median 21.2mg/kg. The concentration of Hg ranged from 0.7 to 0.9 mg/kg with a mean 0.8mg/kg and median 0.8 mg/kg.

Heavy metals have a specific importance in ecotoxicology owing to their long bio-accretion in the food chain and they are non-degradable and unceasingly augmenting in the environment [22, 23]. Most likely, such contamination levels resulted from the long-term environmental loaded by the iron and steel production. It shows on the anthropogenic pollution of industrialized soils in surrounding of U. S. Steel Košice.

3.1. Phytotoxicity tests

Tests with heavy metal-rich soils (1AA-8GA) revealed inhibitory impact of heavy metals (Cr, Cd and Co) for the Sinapis alba (Figure 1-2). The outcome of the seed germination inhibition in Sinapis alba was found into 50% for all industrial soils (1AA-8GA), excepting from 7GA-U.S.S.plant main gate soil (80%). No seed germination inhibition was observed in control soils. The average percentage of growth inhibition for Sinapis alba was observed between 21% - 50% in the seven soils from the Košice area and for 7GA-U.S.S.plant main gate soil was found up to 75%. Soil samples around Košice (1AA-8GA) showed growth inhibition percentage lower than of germination inhibition. Thus, the results of phytotoxicity tests were consistent with the chemical data which are shown in Table 1.

The system of toxicity classification was used to estimate the soil toxicity: PE (percent toxic effect) < 20%, no significant toxic effect (class I), 20% < PE <50% significant toxic effect (class II), 50% <
PE < 100% significant toxic effect (class III), PE-100% (single test - class IV), high acute hazard; PE-100% (all tests - class V), very high acute hazard [11, 24].

Table 1. Statistical summary of metal concentrations (mg/kg) in suburban soils from the Košice area.

|          | Cr  | Co  | Cd  | Hg  |
|----------|-----|-----|-----|-----|
|          | N=34|     |     |     |
| 1AA      | Mean| 49.4| 37.9| 8.9 | 0.1 |
| U.S.S.   | Min | 30.0| 35.0| 8.1 | 0.1 |
| slag heap| Max | 69.0| 40.0| 10.0| 0.1 |
|          | Median| 50.5| 38.0| 8.9 | 0.1 |
|          | St.Dev.| 11.6| 1.6 | 0.6 | 0.0 |
|          | Mean | 156.3| 54.9| 1.8 | 0.1 |
| 2AA      | Min | 135.0| 52.0| 1.5 | 0.1 |
| Gomboš   | Max | 171.0| 58.0| 2.1 | 0.2 |
|          | Median| 157.0| 55.0| 1.8 | 0.1 |
|          | St.Dev.| 11.2| 1.8 | 0.2 | 0.0 |
| 3AA      | Mean| 63.2| 32.8| 4.4 | 0.3 |
| U.S.S.   | Min | 53.0| 30.0| 4.0 | 0.1 |
| plant west| Max | 73.0| 36.0| 4.9 | 0.6 |
|          | Median| 61.5| 32.5| 4.3 | 0.3 |
|          | St.Dev.| 6.3 | 1.8 | 0.3 | 0.2 |
| 4AA      | Mean| 81.5| 33.7| 6.8 | 0.1 |
| Pereš south| Min | 69.0| 31.0| 6.1 | 0.0 |
|          | Max | 92.0| 37.0| 7.9 | 0.1 |
|          | Median| 82.5| 33.0| 6.9 | 0.1 |
|          | St.Dev.| 7.7 | 1.9 | 0.5 | 0.0 |
| 5GA      | Mean| 84.2| 38.8| 1.6 | 0.1 |
| U.S.S.   | Min | 79.0| 36.0| 1.3 | 0.1 |
| slag heap| Max | 90.0| 41.0| 2.0 | 0.1 |
|          | Median| 85.5| 39.0| 1.6 | 0.1 |
|          | St.Dev.| 3.9 | 1.6 | 0.2 | 0.0 |
|          | Mean | 69.7| 22.5| 1.9 | 0.3 |
| 6GA      | Min | 64.0| 20.0| 1.6 | 0.3 |
| Gomboš   | Max | 75.0| 25.0| 2.4 | 0.4 |
|          | Median| 69.5| 22.5| 1.9 | 0.3 |
|          | St.Dev.| 3.1 | 1.8 | 0.2 | 0.0 |
| 7GA      | Mean| 274.7| 38.2| 21.3| 0.8 |
| U.S.S.   | Min | 268.0| 35.0| 20.7| 0.7 |
| plant main| Max | 288.0| 41.0| 22.1| 0.8 |
|          | Median| 273.5| 38.5| 21.2| 0.8 |
|          | St.Dev.| 5.8 | 1.7 | 0.5 | 0.0 |
|          | Mean | 105.1| 29.1| 3.3 | 0.1 |
| 8GA      | Min | 99.0| 26.0| 2.9 | 0.1 |
| Pereš south| Max | 112.0| 31.0| 3.9 | 0.1 |
|          | Median| 105.5| 29.5| 3.2 | 0.1 |
|          | St.Dev.| 4.1 | 1.7 | 0.3 | 0.0 |
|          | Mean | 83.5| 6.5 | 2.1 | 0.1 |
| CS       | Min | 79.0| 5.0 | 1.8 | 0.1 |
| Control soil| Max | 89.0| 8.0 | 2.6 | 0.1 |
|          | Median| 83.5| 6.5 | 2.1 | 0.1 |
|          | St.Dev.| 2.9 | 1.0 | 0.3 | 0.0 |
| LV       |     | 90  | 20  | 1   | 0.75|

U.S. Steel-plant-U.S.S.; Agricultural area-AA; Grass-plot area-GA; Limit value (Low No. 220/2004-2)-LV; Standard deviation-St. Dev; Minimum-Min; Maximum-Max.
The data of potential toxicity (effects) were described follow: agricultural soils 1AA-4AA (PE=31-41) and grass-plot soils 5GA, 6GA and 8GA (PE=31-34) belonged to Classes II (low significant toxic effect). The grass-plot soil 7GA (PE=66) belonged to Classes III (significant toxic effect). This sample is characterized by a very high concentration of heavy metals (Cd, Co, Cr and Hg). The potential toxic effects associated with heavy metals due contamination of industrial soils were in the order: 7GA>2AA>5GA>1AA>8GA>3AA>4AA>6GA.

![Figure 1](image1.png)

**Figure 1.** Germination index (ISG) of the *Sinapis alba* in the five series of the industrial and control soils (CS).

![Figure 2](image2.png)

**Figure 2.** Germination index (IRG) of the *Sinapis alba* in the five series of the industrial and control soil (CS).

### 3.2. Earthworm avoidance behaviour response tests

Pearson matrix correlation coefficients can point on relation between toxicity to organisms and metal concentration in the soils [25]. The distribution of the worms found in the double control was within the range 10–90 % for all areas of the agricultural soils and grass-plot soils, for after 48h (Figure 3). The significant avoidance by *Dendronaena veneta* was up to 100% in soils of the areas Gomboš and from the main gate of the U. S. Steel-plant, Košice.

Correlation matrix of the industrial soils between the percent inhibitions of seed germination of *S. alba* (ISG) / the percent of growth inhibition of *S. alba* (IRG) and Avoidance percentages of the *D. veneta*, and heavy metals were showed in Table 2. Asterisks indicate a significant avoidance using Pearson's matrix correlation. The calculated Pearson’s correlation of metals indicates a strong positive correlation ($r^*=0.66$) among the percent inhibitions of seed germination of *S. alba* (ISG) and
earthworm avoidance for 8GA-Pereš south soils (Grass-plot soils). A very strong correlation (r' = 0.78) is between the percent of growth inhibition of S. alba (IRG) and avoidance percentages of the D. veneta for 3AA-U.S.S. plant west (Agricultural soils). Correlation matrix of this relationship shows that there is a positive correlations (Hg-ISG r' = 0.55 and Cr-ISG (r' = 0.43)/Cd-IRG (r' = 0.63)) between the percent inhibitions of seed germination (ISG) / of growth inhibition (IRG) of S. alba and heavy metals for 1AA-U.S.S.slag heap (Agricultural soils). The correlation matrix indicated serious negative correlation between avoidance tests of the D. veneta and concentration of heavy metals: Hg (r' = -0.88) in 2AA-Gomboš (Agricultural area); Cd (r' = -0.62) for 1AA-U.S.S. slag heap (Agricultural area), Cr (r' = -0.59) and Co (r' = -0.52) in 5GA-U.S.S. slag heap (Grass-plot area) (Table 3). The values of Pearson’s correlation coefficients are over 0.5 and -0.5. The elements show strong correlation coefficients and it indicates the degree of urban contamination (i.e. Cd, Co and Cr). These significant correlations reveal their common sources U. S. Steel-plant, Košice. The other sources of heavy metals may be from pesticides and fertilizers used for cultivation of crops [26]. The studied results may provide a baseline data for soil contamination and information about ecosystem in urban regions subjected to emission of industrial sources.

Table 2. Correlation matrix of the industrial soils between the percent inhibitions of seed germination of S. alba (ISG) / the percent of growth inhibition of S. alba (IRG) and Avoidance percentages of the D. veneta, and concentration of heavy metals.

| A-test (N=34) | 1AA Phyto | 2AA Phyto | 3AA Phyto | 4AA Phyto | 5GA Phyto | 6GA Phyto | 7GA Phyto | 8GA Phyto |
|--------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| 1AA-SGA ISG  | 0.48      | 0.40      | -0.01     | -0.40     | 0.09      | 0.19      | 0.07      | **0.66**  |
| 1AA-SGA IRG  | **-0.54** | -0.43     | **0.78**  | -0.13     | 0.09      | 0.25      | 0.12      | -0.40     |
| Cr-ISG       | 0.43      | 0.200     | 0.39      | **-0.52** | -0.09     | **-0.53** | -0.18     | -0.19     |
| Cr-IRG       | -0.31     | 0.008     | **0.83**  | 0.45      | 0.35      | 0.19      | -0.44     | 0.38      |
| Co-ISG       | 0.16      | 0.230     | 0.51*     | -0.38     | -0.18     | -0.08     | -0.12     | -0.02     |
| Co-IRG       | 0.08      | -0.080    | **0.60**  | 0.29      | 0.33      | 0.30      | -0.34     | -0.02     |
| Cd-ISG       | -0.20     | 0.260     | 0.10      | 0.44      | -0.22     | -0.28     | 0.20      | -0.17     |
| Cd-IRG       | **0.63**  | -0.030    | -0.15     | 0.03      | 0.27      | -0.42     | 0.01      | 0.26      |
| Hg-ISG       | **0.55**  | -0.330    | 0.11      | 0.07      | -0.42     | 0.36      | -0.33     | -0.16     |
| Hg-IRG       | 0.04      | **0.54**  | 0.13      | 0.30      | 0.23      | -0.20     | -0.67     | -0.32     |

*Correlations are significant at over 0.5 and -0.5 levels; A-test – Avoidance percentages; Phyto - Phytotoxicity tests.

Effect of metals on earthworms mortality (ANDE), and correlation matrix of the industrial soils between Avoidance tests of the D. veneta and heavy metals are in Table 3. Low mortality effects were recorded in the tests with D. veneta. The contaminated soils with lower (sub-lethal) pollutant concentrations require more sensitive test methods such as behavioral tests (Avoidance behaviour response tests) in their risk assessment.

Table 3. Effect of metals on earthworms mortality (ANDE), and correlation matrix of the industrial soils between Avoidance tests of the D. veneta and concentration of heavy metals.

| (N=34) | 1AA A-test | 2AA A-test | 3AA A-test | 4AA A-test | 5GA A-test | 6GA A-test | 7GA A-test | 8GA A-test |
|--------|------------|------------|------------|------------|------------|------------|------------|------------|
| Mortality | 0          | 0          | 1          | 0          | 0          | 0          | 1          | 0          |
| Cr     | 0.16       | -0.18      | 0.47*      | 0.16       | **-0.59**  | 0.01       | -0.28      | 0.29       |
| Co     | 0.45       | 0.08       | 0.14       | **0.57**   | **-0.52**  | 0.46*      | -0.46*     | 0.22       |
| Cd     | **-0.62**  | 0.04       | 0.21       | -0.36      | -0.17      | -0.35      | -0.01      | 0.33       |
| Hg     | 0.44*      | **-0.88**  | 0.22       | 0.12       | **0.49**   | 0.18       | -0.29      | -0.06      |

A-test- Avoidance behaviour response; (ANDE-absolute number of dead earthworms); *Correlations are significant at over 0.5 and -0.5 levels
The data indicate that under conditions of the experiment, earthworm movement from the contaminated side of the test chamber to the uncontaminated (control soil) side occurred as a result of the presence of heavy metals (in Figure 3). This movement occurred at higher concentrations for the Cr, Cd, and Co studied, and low Hg concentration for the samples 3AA-Gomboš and 4AA-Pereš south (Agricultural soil), and 6GA-Gomboš (Grass-plot soil). Results showed that heavy metals are toxic in different concentration ranges for these organisms.

![Figure 3](image-url)

**Figure 3.** Avoidance percentages, behaviour responses of *Dendrobaena veneta* for each tested soils.

### 4. Conclusions

The present study investigated the potential effect of heavy metals in urban soil on biomarker of earthworm, *Dendrobaena veneta* and on the phytotoxicity tests, *Sinapis alba* and quantify potential environmental risks to ecosystem. The statistical analysis results show that the industrial soils are significantly contaminated with heavy metals in the order Cd< Co<Cr for all agricultural soils and grass-plot soils 1AA-8GA and pointed to the highest soil ecotoxicity in the grass-plot soil 7GA. The potential toxic effects associated with heavy metals due contamination of industrial soils were in the order: 7GA>2AA>5GA>1AA>8GA>3AA>4AA>6GA. The significant avoidance by *Dendronaena veneta* were up to 100% in soils of areas Gomboš (2AA and 6GA) and from the west (main gate) of the U. S. Steel-plant (3AA and 7GA), Košice. Results show that pollution mainly comes from the mainly around vicinity of U.S. Steel Košice and agricultural activities have a certain impact too.

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