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Environmental risk assessment of metal-contaminated areas using different bioassays

Oľga Šestinová✉, Jozef Hančuľák and Lenka Findoráková

Department of Environment and Hygiene in Mining, Institute of Geotechnics, Slovak Academy of Sciences, Watsonova 45, Košice 04001, Slovak Republic

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Abstract

Mining activities in the areas Krompachy and Rudňany-Markušovce were focused on mining and processing of copper and mercury ore and left harmful effects on the region of Eastern Slovakia. The aim of this study is using different screening methods (XRF, Phytotoxkit and earthworm bioassays) for environmental risk assessment of metal-contaminated areas. Elemental analysis by X-ray fluorescence spectrometry indicated severe pollution of studied soils by Cu, Ni, As and Hg, which exceeded limit values. Significant positive correlation is found between Pb and Zn occurrence in the agricultural soil from Krompachy: Kluknava, and for the contents of particular metals in soil from permanent grass vegetation in Kolinovce locality, namely between Pb and Ni, Pb and Zn, and between Hg and Zn contents. A 7-day bioassay and avoidance test with the *Dendrobaena veneta* was used to assess the environmental risk of heavy metals in soils. The earthworms mortality was very little influenced by metals in Krompachy soils, but rather affected by Rudňany soils tailing. Phytotoxkit results for soils from Krompachy showed inhibition in germination by 32 % and 29 % for *Sinapis alba* and *Lepidium sativum*, respectively. Results of the average percentage of growth inhibition by *Lepidium sativum* was 28 % and 24 % for *Sinapis alba*. On the other hand, soil samples from Rudňany tailing showed 56 % of germination inhibition by *Sinapis alba*, and 49 % for *Lepidium sativum*, respectively. Results of the average percentage of grow the inhibition by *Lepidium sativum* was 48 %, and 52 % for *Sinapis alba* Rudňany tailing soils. The significant results ($P < 0.05$) of the avoidance percentages of *Dendrobaena veneta* for tested soils were within the range 80 – 100 % in soils Rudňany-Markušovce tailing after 48 h. The variable toxicity of contaminated soils demonstrated the efficiency and usefulness of the Phytotoxkit and earthworm bioassays as a useful tool for evaluation of soil ecotoxicity. The results supported the expected negative impact of the soil samples on the region Eastern Slovakia.

Introduction

Soil is a dynamic and complex system that provides a habitat for different microorganisms, flora, animals, and humans. These days' soils are exposed to anthropogenic contamination with heavy metals that create a risk in many ecosystems as well as human health. Slovakia is note due to metal ore mines and their processing. The soil chemical reduction by heavy metal contamination, especially in Eastern Slovakia is an actual issue of societal matter. From the occurrence point of view of ores and industrial mining, it is one of the most significant geological formations

✉ *Corresponding author:* sestinova@saske.sk

in Slovakia (Demková *et al.* 2017; Maity *et al.* 2018; Šestinová *et al.* 2019a). Mining activities in the region Eastern Slovakia, Krompachy and Rudňany-Markušovce are focusing on copper and mercury ore mining and processing. Hence, the determination of the total heavy metal matter is not adequate to score the environmental problem, which is inseparable to a contaminated soil. For assessment of soil quality, bioassays could be effective tools to scale the potential contaminants toxicity aimed at bioavailability fraction (Hund-Rinke *et al.* 2003; Loureiro *et al.* 2005; Šestinová *et al.* 2019c). Phytotoxkit (Phytotoxkit 2004) is an alternative test procedure which is giving estimation of the chemical compounds biological effects on plants.

Phytotoxicity bioassays with a higher plant collated on seed germination and root elongation growth measurements were conducted with different terrestrial plant species (Wang 1991; Garcia-Lorenzo *et al*. 2009). Data on phytotoxicity studies were thought over the toxicity evaluation of trade chemicals (Günter and Pestemer 1990), industrial effluents, dangerous wastes, and leachates (Vasseur *et al.* 1998; Czerniawska-Kusza and Kusza 2011). Recently, phytotoxicity tests with terrestrial plants are accepting increasing attention also in assessing soil toxicity. Given to its short-exposure periods and its high sensitivity, macrobiotics was successfully used as appropriate help for screening; hence they contribute to a responsible risk assessment for soil environments (Persoone *et al*. 2003). Earthworms are commonly used as model organisms to evaluate the ecological risk of various pollutants on soil ecosystems since are considered as fundamental organisms for proper soil functionality (Aldaya *et al.* 2006; Latha and Basha 2019).Furthermore, as major agents of the soil fauna biomass they contribute to soil aeration and drainage (Davies *et al*. 2003) as well as decomposition of organic matter that can be reduced as a consequence of pollution. Ability of organisms to evade contaminated soils serves as an indicator of soil ecotoxicity. Nannoni *et al.* (2014) has reported on variable bio-concentration of metal trace elements in earthworms at urban, peri-urban and garden levels depending on extent of pollution. Avoidance tests performed on soils from contaminated sites usually score and the response of organisms to a combination of different soil properties (e.g. structure, content of organic matter) with the effects of the contaminants. Soil properties outside the range of physiological requirements of the test species strongly influence the avoidance response to pollutants. Avoidance tests with most abundant soil organisms can be used as an early screening tool assessment. This study is devoted to evaluate the environmental risk in study soils using tests of ecotoxicity, Phytotoxkit microbiotest, earthworm acute toxicity and avoidance tests. The presented approach contributes to the future resolution of a local pollution problem in metal-contaminated environments.

Experimental

Samples and analysis

Soils were collected from two areas Krompachy (KR) and Rudňany-Markušovce (RM). Mining activities by the metallurgical treatment of complex metals and copper ores left negative effects on the region Eastern Slovakia, Krompachy and Rudňany- Markušovce tailing (Angelovičová *et al.* 2015; Šestinová *et al.* 2019a). The Markušovce tailing is situated between the Markušovce and Rudňany villages (Spišsko-Gemerské Rudohorie Mountains). Its construction and operation are connected with former and current mining activities on the Rudňany and Poráš-Zlatník polymetallic ore deposits. The thickness of deposited materials varies in the dependence on initial ground levels and it may achieve maximally about 38 m^3. Recently, the settling pit is in operation by the SABAR, Ltd. Markušovce. Altogether 9 800,000 tons of the tailings of so-called flotation sands at the density of 1.59 $t.m^{-3}$ and a total volume of 6 200,000 m^3 are deposited in the settling pit (Jakabský *et al.* 2010; Hredzák *et al.* 2019). In the year 2015, the two sampling sites were localised in the surrounding Krompachy town sampled were agricultural soil in Kluknava: (KR-AS) and permanent grass vegetation soil in Kolinovce: (KR- PGVS). The soils were collected from a depth of 20 – 40 cm into bags. Soils were homogenized, dried at room temperature, and sieved through 2-mm sieve (weight 5 kg). Trough X-ray fluorescence spectrometer model XEPOS 3 the concentrations of Cu, Zn, Ni, Pb, As and Hg in soils and control reference soil (CRM-TM52) were measured. All the analyzed samples were conducted in triplicate.

Soil phytotoxicity

Evaluation of soil phytotoxicity was based on germination and seedling growth of the two terrestrial plants (mustard *Sinapis alba* and garden cress *Lepidium sativum*), by use the Phytotoxkit microbiotest (OECD 208). The soil was covered by the filter plate and after that seeds of the plant were placed on top of the filter in a single row. The test plates with the cover, they were placed vertically and incubated for 72 h at 25 °C. Five replicates were performed for the sample set. Root growth inhibition was measured after 72 h. The test was considered to be valid if the number of germinated seeds in the control which was at least 90 %. Pictures of the test plates were analyzed through the program Image Tools. The percent inhibition of seed germination (*ISG*) and inhibition of root growth (*IRG*) for the plant were calculated using (Eq. 1):

$$ISG(IRG) = \frac{A - B}{A} \times 100 \quad [\%] \qquad (1)$$

where *A* is the mean seed germination or root length in the control (mm); and *B* is the mean seed germination or root length in the test soil (mm).
The system of toxicity classification generated by Persoone *et al*. 2003 was used to forecast the soil toxicity: PE (percent toxic effect) < 20 %, no significant toxic effect, **class I**, no acute hazard; 20 % < PE < 50 % significant toxic effect, low toxic sample, **class II**, low acute hazard; 50 % < PE < 100 % significant toxic effect, toxic sample, **class III**, acute hazard; PE-100 % (single test), **class IV**, high acute hazard; PE-100 % (all tests), **class V**, very high acute hazard.

Acute bioassays with Dendrobaena veneta

The acute bioassays were based on Earthworm acute toxicity tests, (OECD 207). From a local supplier adult earthworms were purchased. Ten earthworms (and C.W. – control worms) were placed to a plastic box (9 cm × 9 cm × 3 cm) control worms were added to 100 g of CRM soil. Distilled water was added and the boxes were maintained at laboratory temperature for 7 d. Earthworms' mortality for each soil was estimated and compared with corresponding control. Finally, the earthworms were lyophilized; the samples were mineralized and analyzed by atomic absorption spectrometry (AAS Variant, Australia) to determine the metals concentrations in warm tissues (Šestinová *et al*. 2019b).

Avoidance bioassays with Dendrobaena veneta

The avoidance tests with earthworms were based on ISO guideline 17512-1/2008 – Avoidance test for determining the quality of soils and effects of chemicals on behaviour: Test with the earthworms. Every replicate was composed of a plastic box: 23 cm × 13 cm × 6 cm. The box was distributed into two parts by a divider. Every part was filled with 250 g dry weight of one of the three test soils (KR-AS, KR-PGV, RM) and a control soil (CRM) on the opposite. Later, the divider was eliminated and 10 adult earthworms were located onto the middle line. The test containers were over casted by a plastic wrap perforated with pinholes to facilitate air exchange. The test containers were incubated at 20±2 °C and a 16 : 8 h light : dark photoperiod for 48 h. Subsequently, the vessels were divided again with the divider at the end of the period, and the earthworms were counted on each side of the replicates. In the midline of the test container an earthworm found was counted as 0.5 earthworms. Earthworms found on the underside of the lid of the test were counted as dead. Soil pH was measured at the beginning and the end of the test period. The avoidance rates of worms to different soils were calculated according to Eq. 2, and expressed as a percentage:

$$R\,(\%) = \frac{[(C - T)]}{N} \times 100 \quad [\%] \qquad (2)$$

where *R* = avoidance; C = number of worms in the control (C_0) condition; *T* = number of worms in each dose in the same soil; *N* = total number of worms. Positive values account for avoidance of worms in test soil, while neutral or negative responses represent indifference or preference of the test substance (Hund-Rinke *et al*. 2003).

Table 1. Basic properties of analyzed soils samples from agricultural (KR-AS) and permanent grass vegetation soils in Krompachy (KR-PGVS), and from Rudňany-Markušovce (RM).

	Soils		
	KR-AS	**KR-PGVS**	**RM**
pH/ H_2O	6.5	6.9	8.1
pH/ KCl	5.2	7.2	7.8
Eh [mV]	582	580	686
Dry weight [%]	97.9	94.3	99.6
Organic matter dry weight [%]	5.6	7.3	9.8
Grain size [%]			
Sand	3.7	4.5	4.2
Silt	30.2	24.7	27.2
Clay	66.1	70.8	68.6

Dry weight (STN EN 75791), Organic matter dry weight (STN EN 12879).

Statistical analysis

Statistical analyses were done through SPSS ver. 9.0 software. By the Pearson matrix correlation the statistical dependence between total metal concentrations was evaluated. Correlation analysis was used as a comparison of the potential relationships among the data. The certified river sediment (LGC6187) was used to validate data. Control earthworms (CRM) fended in non-contaminated soil was used.

Results and Discussion

Soil physical and chemical properties, including grain size of the studied soils, are presented in Table 1. We recorded acidic pH values for the soil samples (KR-AS), near-neutral pH values for the soils from KR-PGVS and near-alkaline pH values for the soil samples from RM. The organic matter dry weight of all soils ranged between 5.6 to 9.8 %. The soil organic matters content could reduce metal concentrations in soil solution, and then, their potential availability and toxicity to earthworms (Lukkari *et al.* 2006; Šestinová *et al.* 2019b). All soil samples included the sand, silt and many clay fractions (66.1 – 70.8). Soil types were silty-clay texture for all soil samples. Some studies have demonstrated that variations on pH, electric conductivity, salinity, redox potential, cation exchange capacity (CEC), clay minerals, texture, permeability, porosity and moisture content influence the behaviour of metals in soil (Das *et al.* 2011; Angelovičová *et al.* 2015; Šestinová *et al.* 2019a). Due to the heavy metal limits in the Slovakia soils (Law No. 220/2004), we found quite extensive contamination with Cu, Hg, As, and Ni in all studied localities (KR-AS, KR-PGVS and RM) (Table 2). It was found that after 7-days exposure, earthworms in some instances caused decrease of metal concentrations in contaminated soils; such drop was estimated between 5 – 20 % for Cu, 4 – 19 % for Hg, 5 – 17 % for As, 4 – 15 % for Pb, 7 – 14 % for Ni and 2 – 11 % for Zn, for all studied soils. Most processes, including direct dumping of wastes from ore extraction and manufacturing, may bring heavy metals into the surface soil. Usually, soil properties determine the bioavailability of toxic heavy metals (Šestinová *et al.* 2017; Maity *et al.* 2018; Bravo *et al.* 2019).

Acute toxicity tests results and the metals concentrations of the *D. veneta* tissue are demonstrated in Table 2. The earthworms mortality (*n*) was little effected by Krompachy soils, and rather influenced by Rudňany tailing soils. The higher pollutant concentrations (heavy metals) may be evaluated by the acute test, which propose dose-dependent effects on mortality. Less polluted soils require for reliable risk assessment more sensitive assays such as behavioral tests (Avoidance bioassays).

Pearson correlation analysis among heavy metals in the soils is demonstrated in Table 3. Statistical correlations at the levels $P < 0.05$ and $P < 0.01$ were considered significant. Significant positive correlation was founded between Pb and Zn ($r = 0.68$, $P < 0.05$) in the KR-AS soil, for the KR-PGVS soil was found between Pb and Ni ($r = 0.86$, $P < 0.05$), Pb and Zn ($r = 0.66$, $P < 0.05$), and Hg and Zn ($r = 0.79$, $P < 0.05$) (Table 3). Significant positive correlation was identified among As and Zn ($r = 0.90$, $P < 0.05$) and Hg and Pb ($r = 0.65$, $P < 0.05$) for the RM-tailing soil (Table 3). Significant correlation was not be identified for As in the Krompachy soils (KR-AS and KR-PGVS). To know the heavy metals source the correlation analysis is a suitable way. Many authors dealing with the Eastern Slovakia environmental pollution has founded that the soil environment contamination by arsenic is related not only to anthropogenic impact but also to

Table 2. Heavy metal content and corresponding worm mortality (*n*) in soils from the region of Eastern Slovakia (average ± standard deviation).

Locality	Cu [mg.kg^{-1}]	Zn [mg.kg^{-1}]	Ni [mg.kg^{-1}]	Pb [mg.kg^{-1}]	As [mg.kg^{-1}]	Hg [mg.kg^{-1}]	Mortality [%]
	Metal concentration in soils						
No treatment							
KR-AS	172±56	220±54	62±14	75±25	50±12	3.2±1.0	–
KR-PGVS	325±74	478±82	81±14	109±9	75±19	20.4±2.5	–
RM	634±98	35±15	98±21	31±5	104±28	82.5±8.1	–
CRM	3.2±4.0	6.5±5.0	11±2	26±8	1.4±1.0	0.1±0.0	–
Metal concentration in soils after 7-days bioassay							
KR-AS	156±49	215±36	53.2±8.0	71.5±19	46±6	2.61±1.71	*n* = 2
KR-PGVS	309±30	452±62	75±15	104±11	71±13	18.5±1.0	*n* = 4
RM	511±99	31±10	91±21	26.5±3.0	86.5±19.0	79.2±0.1	*n* = 6
CRM	2.8±1.0	6.0±2.0	12±4	24±3	0.9±1	0.10±0.01	*n* = 1
Metal concentration in D. veneta tissues/7-days							
KR-AS	12.1±15	102.5±17.5	2.4±1.1	7.8±9.5	6.3±2.1	0.12±0.03	–
KR-PGVS	17.1±10.0	122±19	2.6±1.3	5.6±1.6	7.6±2.5	0.54±0.35	–
RM	24.7±21	105±11	19.1±11.5	14.2±4.2	5.2±1.9	2.27±1.15	–
C. W.	4.3±2	1.5±1.2	2.1±0.8	2.4±0.2	0.4±0.0	0.47±0.10	–
Limit	*70*	*200*	*60*	*115*	*30*	*0.75*	–

KR-AS – agricultural soil and KR-PGVS – soils from permanent grass vegetation both from Krompachy, tailing in Rudňany-Markušovce; Control reference material – CRM; Law No. 220/2004 – *Limit*; Control worm – C.W.

the geochemical effects of mineralized zones (Hronec *et al.* 2008; Kučerová *et. al.* 2014; Angelovičová *et al.* 2015; Demková *et al.* 2017).

The individual heavy metals presence is caused by mining and smelting activities, as confirmed by significant positive correlations.

Table 3. Correlation relationship between heavy metals in soils from studied localities.

	Zn	Ni	Pb	As	Hg
Agricultural soil: Krompachy					
Cu	0.072	-0.37	0.2	0.3	0.06
Zn		0.37	**0.68**	-0.31	0.09
Ni			-0.08	0.16	0.22
Pb				**-0.73**	0.6
As					-0.71
Permanent grass vegetation soil: Krompachy					
Cu	-0.37	0.16	0.059	-0.33	-0.39
Zn		**0.57**	**0.66**	0.25	**0.79**
Ni			**0.86**	0.39	-0.007
Pb				-0.01	0.22
As					-0.16
Tailing soil: Rudňany-Markušovce					
Cu	0.41	-0.42	**-0.71**	**0.43**	-0.22
Zn		-0.86	-0.16	**0.9**	-0.09
Ni			0.18	**-0.83**	0.35
Pb				-0.26	**0.65**
As					-0.4

Impact on plant performance was further tested. Phytotoxkit results for soils from Krompachy showed inhibition in germination by 32 % and 29 % for *Sinapis alba* and *Lepidium sativum*, respectively (Fig. 1). Results of the growth inhibition average percentage by *Lepidium sativum* was 28 %, and 24 % for *Sinapis alba*. On the other hand, soil samples from Rudňany tailing showed 56 % of germination inhibition by *Sinapis alba*, and 49 % for *Lepidium sativum*. Results of the growth inhibition average percentage by *Lepidium sativum* revealed 48 % and 52 % for *Sinapis alba* for Rudňany tailing soils, see in Fig. 2. Soils of the RM-tailing have high As (104 mg.kg^{-1}) and Hg (82 mg.kg^{-1}) contents. The growth inhibition average percentage for *L. sativum* was 28 %, 24 % for *S. alba* in the two soils studied from the Krompachy area, as shown in Fig. 2.

The growth inhibition results showed that the potential toxicity is lower for the Krompachy soils. RM-tailing showed growth inhibition percentage lower than 50 % and the values are lower than those for germination inhibition. Many agents that might have an impact on this plant response are present. The elements with similar physico-chemical properties might interfere in enzymatic pathways, compete during transport and affect

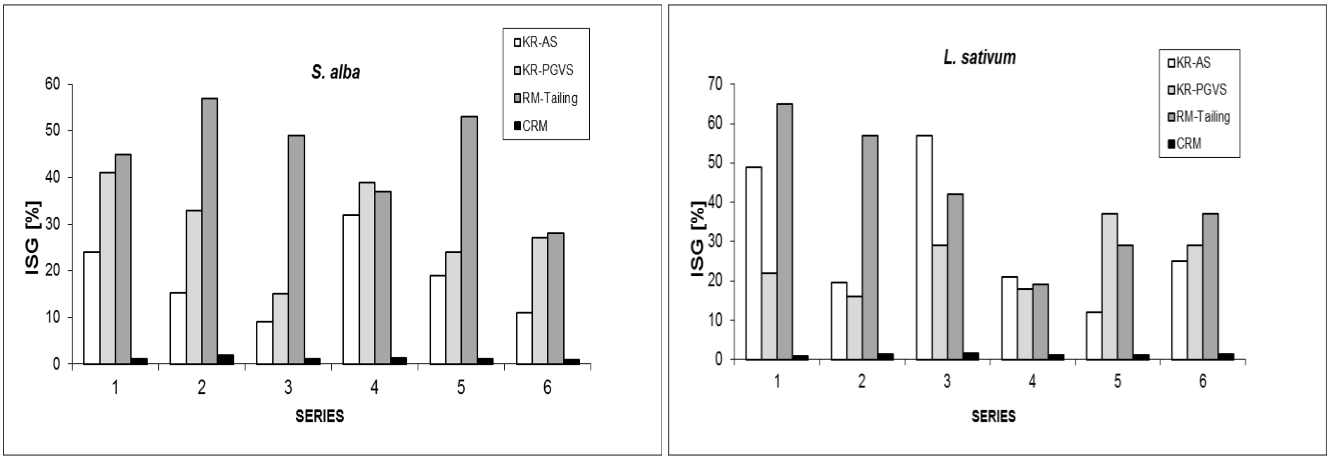

Fig. 1. Index of inhibition in seed germination (ISG, in %) of *Sinapis alba* and *Lepidium sativum* in the six series of the soil samples, Krompachy soils (KR-AS and KR-PGVS), Rudňany tailing soils (RM-Tailing) and Control reference sample (CRM). Data represent average of n = 5.

accumulation rate (Valerio *et al.* 2007; Fu *et al.* 2019). Fargašová (1999) reported a strong antagonistic effect between Ni and elements like Mo, Cu, Mn, and described a significant stimulatory effect on *S. alba* seedlings cultivated hydroponically.

The potential toxicity data were described by the use of the toxicity classification system proposed by Persoone *et al.* 2003. The Control reference sample (CRM) belonged to Class I (No acute hazard).All soil samples (KR-As and KR-PGVS) belonged to Classes II (Slight acute hazard) and soil samples (RM-tailing) belonged to Classes III (Acute hazard). This sample are characterized by a very high total concentration of heavy metals (Cu, Ni, As and Hg).

The results of the avoidance percentages of *Dendrobaena veneta* for three tested soil are shown in Fig. 3. Avoidance tests with earthworms were controlled by dual combinations of each test soil *vs.* the corresponding reference soil. The test was performed in a two chamber system. Soils were not acutely lethal to earthworms. The distribution of the worms found in the double control was within the range 10 – 40 % for soils from KR-AS, while from KR-PGVS locality they were 40 – 80 % after 48 h. On the contrary, significant (P < 0.05) avoidance by *D. veneta* was 80 – 100 % in soils from Rudňany–tailing. The habitat soils function was controlled to be limited if less than 20 % of the organisms (on average) were found in the soil. This indicates an impact on properties and deputizes the ‘‘habitat function’’. Soils of limited function are where 80 % of the total animals are found in the control soil and 20 % in the polluted soil (Latha and Basha 2019). Behaviour is a final integrated result of sensory, hormonal and metabolic processes.

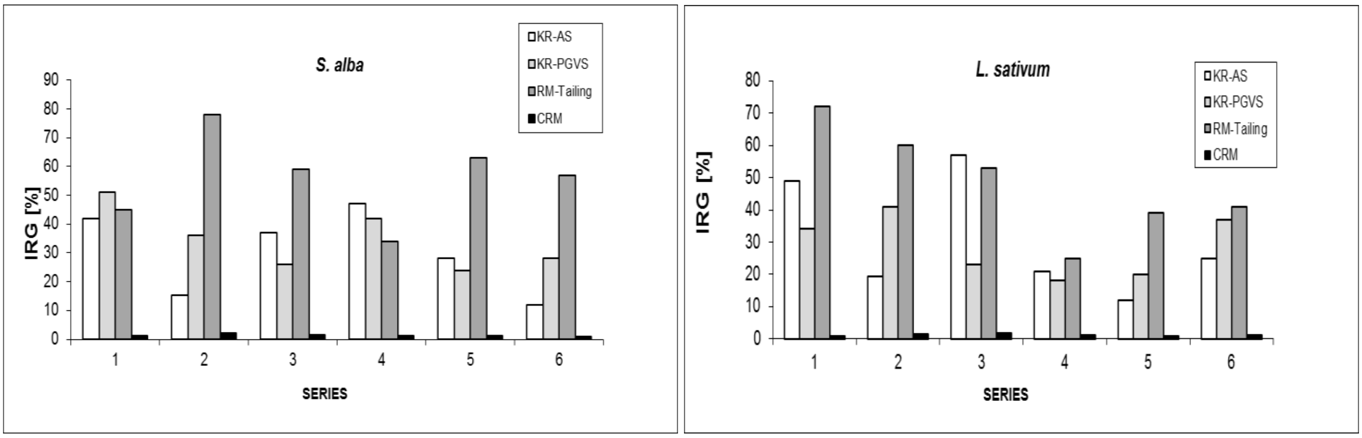

Fig. 2. Index of root growth inhibition (IRG, in %) of the *Sinapis alba* and *Lepidium sativum* in the six series of the soil samples, Krompachy soils (KR-AS and KR-PGVS), Rudňany tailing soils (RM-Tailing) and Control reference sample (CRM). Data represent average of n = 5.

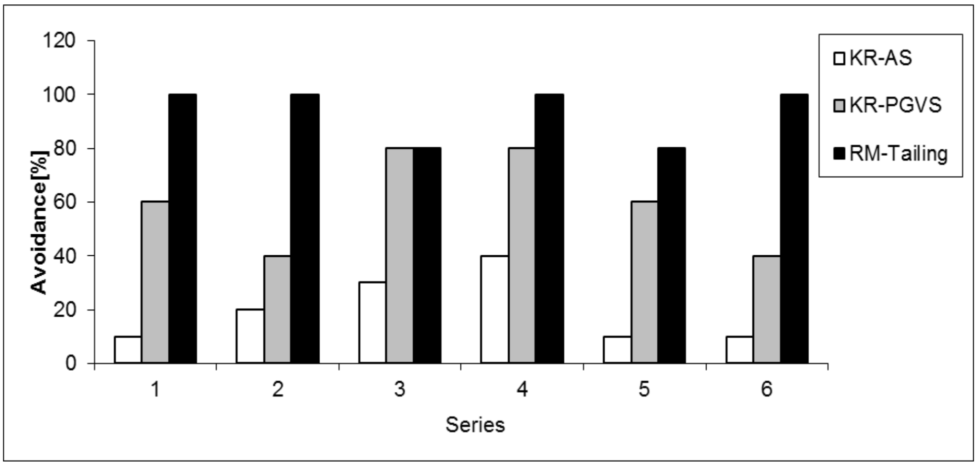

Fig. 3. Avoidance responses of the earthworm (*D. veneta*) in the six series of three different soil samples, Krompachy soils (KR-AS and KR-PGVS), and Rudňany tailing soils (RM-Tailing). Data represent average of $n = 5$.

The habitat function of soil is involved in the interaction with biotic and abiotic components of the environment (Maity *et al.* 2018). In our case, when we compared all the results of the used bioassays, we obtained agreement in the evaluations so that the highest soil ecotoxicity was demonstrated in soil from Rudňany-Markušovce Tailling. Capowiez *et al.* (2006) suggests that behaviour seems to be a promising (efficiency) biomarker in earthworm studies, as it may give various endpoints which can perhaps be linked to soil functioning. Avoidance response tests reflect this behaviour of the earthworms, which works on the principle of preference or avoidance of substrates after a specific exposure period. The sensory cells in the epithelium of mouth region enable the earthworms to avoid adverse habitats (Stephenson *et al.* 1998). According to Manzo *et al.* (2014) a larger tests number with higher sensitivity together with a tailored weights attribution to endpoints and matrices would improve the final site evaluation. The results show that earthworm avoidance behaviour is an ecologically relevant parameter for assessing toxic metal spiked soils. Information combining from screening methods oriented on ecological receptors at risk by using available chemical methods and ecotoxicity tests have great benefit for further research of metal-contaminated sites. Advantage of combined methods is that the results can be compared and statistical analyzed together and so it is better to estimate the risk of ecosystems contamination. Thus, the tools of combined screening with environmental risk assessment in a certain area should be able to indicate effects, but also rapid, easy to apply and cheap (Semenzin *et al.* 2008).

Conclusions

To human health and other organisms the heavy metals contaminated soils are one of the environmental issues considered to be a serious threat. The toxicity and metal bioavailability were studied in soils from Krompachy and Rudňany-Markušovce in Eastern Slovakia. Average Cu, Ni, Hg and As concentrations are well above the limit levels, defining the need to apply a risk assessment procedure in order to determine and quantify potential environmental risks both to ecosystems. All soil samples from Krompachy locality belonged to slight acute hazard (Classes II) and soil samples from Rudňany-Markušovce (RM-tailing) belonged to acute hazard (Classes III). The abundance of the worms found in the double control was within the range of 10 % – 40 %, and for the soils from Krompachy it was 40 % – 80 % after 48 h. On the contrary, significant ($P < 0.05$) avoidance by *D. veneta* of 80 % – 100 % was estimated in the soils RM-tailing. The results of the used bioassays coincided and pointed to the highest soil ecotoxicity soil from Rudňany-Markušovce tailing. Toxicity tests allowed us to carry out a preliminary screening of the contaminated areas ecotoxicological risk. The obtained results show that the Phytotoxkit test with *Sinapis alba* and *Lepidium sativum*, and avoidance test with earthworms (*Dendrobaena veneta)* can be considered as suitable tools in the soil contamination screening evaluation.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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