



Biochar, wood ash and humic substances mitigating trace elements stress in contaminated sandy loam soil: Evidence from an integrative approach

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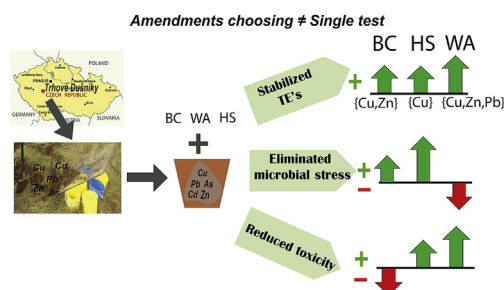
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HIGHLIGHTS

- Wood ash is reliable to decrease the water- and weak acid-extractable Cu, Zn, and Pb soil content.
- Carbonaceous amendments decrease weak acid-extractable Cu, and Zn soil content.
- Wood ash depressed soil enzymes activity.
- Biochar addition may pose a risk to *Eisenia foetida* survival.
- Minor addition of humic substances creates favourable soil condition for enzymes and earthworms.

GRAPHICAL ABSTRACT



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ABSTRACT

We conducted a pot experiment with biochar (BC), wood ash (WA), and humic substances (HS) to investigate their effect on As, Zn, Cu, Cd and Pb mobility in soil, as well as enzyme activities involved in C-, N-, and P-cycles, and *Eisenia foetida* toxicity in multi-contaminated soils. Amendments were dosed to increase soil pH from initial 6.0 to ~6.5 and ~7.0. Applying amendments has revealed that WA significantly immobilized Cu, Zn and Pb, BC – Cu and Zn, and HS decreased solely Cu mobility in soil. The partition indices of Zn, Cu, and Pb, quantitatively describing the bioavailable species of elements in soil, were the lowest for WA. Changes in the water-soluble species of metals were more pronounced than in the exchangeable ones for all amendments. An opposite effect was observed on enzyme activity and earthworm toxicity for the WA and carbonaceous amendments. The BC and HS provided favourable soil conditions to dehydrogenase, β-glucosidase, urease activity and fluorescein diacetate hydrolysis, while WA significantly decreased the activity of all the mentioned enzymes in soil. The results are supported by an enzymes-based weighted mean index, being the highest for BC and HS and the lowest for WA (lower than in the control sample). At the same time, WA was suitable to eliminate the trace elements' stress to earthworms (biomass endpoints and cocoons production). Our data revealed that each amendment has its own advantages and disadvantages. The choice of the most suitable amendment therefore should always be made within an integral approach and based on the purpose of remediation.

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1. Introduction

Trace element pollution of soils is the major concern threatening ecosystems, water bodies, food safety and human health. Therefore, favourable soil conditions are crucial, and the search for the most beneficial amendments is of great importance (Rao et al., 2017). Considerable attention has been paid to waste recycling materials, and several materials have been proposed for soil reclamation, such as wood ash (Demeyer et al., 2001), biochar (Kuppusamy et al., 2016), or humic substances (Perminova and Hatfield, 2005). The primary mechanisms of immobilizing by biochar (BC) in soils include alkalization, enhancement of ion exchange capacity and increment of physical sorption and precipitation (Beesley et al., 2015; Li et al., 2017). The beneficial properties of wood ash (WA) have been linked to its high alkalinity and nutrient concentration (Ca, Mg, P and K) (Mercl et al., 2016). The humic substances (HS) protective effect has been generally attributed to the formation of metal-humic complexes and presence of carboxyl, hydroxyl, and amino groups (Tan, 2014). Overall, the effect of carbonaceous soil amendments was recently reviewed by Ren et al. (2018). BC, WA, and HS having potential to restore the degraded or contaminated soils due to the abovementioned characteristics.

Environmental risk assessment of trace elements (TE) pollution is usually based on the analysis of their available concentrations in soil. However, the chemical analyses fail to reveal the complex interactions between the contaminants and soil environment; the formation of toxic intermediate metabolites or changes in TE mobility may increase soil toxicity during the remediation processes (Manzano et al., 2014). Consequently, the combined use of chemical analyses and biological assays is advantageous as it integrates the biological effects of all compounds present, taking into account the following factors: bioavailability, synergism, or antagonism (Stephenson et al., 2002; Fernández et al., 2005). Thus, the using the soil enzymes activities as screening tools to characterize contaminants in a variety of environmental matrices has become a popular, powerful and reliable tool in the environmental toxicology (Alkorta et al., 2003; Luo et al., 2017). Moreover, earthworms are generally used for toxicological tests as they are in direct contact with soil and are important in terrestrial food webs, soil productivity and fertility (Hirano and Tamae, 2011). In this study, we chose the *Eisenia foetida* earthworm species that is widely used as a model soil organism in research and governmental guidelines (Reinecke, 1992; Environment Canada, 2004).

Several studies reported negative or zero effects of BC, WA and HS on soil biological properties (Björk et al., 2010; Zhang et al., 2014). The reasons for this are diverse. Firstly, in many cases, these products are applied as recommended by manufacturers, sometimes with little or even no knowledge of the optimal rates, timing and methods of application. Secondly, the incorrect choice of product concentrations or disregarding the environmental aspects may contribute to a lack of response to amendments. Therefore, elucidating their specific selectivity to different types of pollution for remediation purposes is in high demand for soil studies of the new millennium. Our previous study (Pukalchik et al., 2017) focused on the effect of BC, WA, and HS amendments on the plant-available TE concentrations, microbial respiration and ecotoxicity (acute toxicity test with *Daphnia magna* and *Sinapis alba*) but the effect of the aforementioned amendments on soil enzymatic activities and soil habitat functions has not been previously evaluated.

The present study focusing on the relationships between water-soluble and exchangeable concentrations of Cu, Zn, Pb, Cd and As, soil enzymatic activities and earthworm's response in highly contaminated sandy loam soil amended with BC, WA, and HS. Hence, our study may establish the knowledge of soil-interlinked

interactions required to manage and improve the waste material amendments used in soil reclamations.

2. Material and methods

2.1. Soils

The soil was sampled from the topsoil layer at the alluvium of the Litavka River in vicinity of the village of Trhové Dušníky (Czech Republic; 49°43'08.0"N 14°00'46.4"E). The history of excessive concentrations of TEs in the soil is associated with mining and smelting activities in this region (Vyslouzilová et al., 2003; Vaněk et al., 2005). The physicochemical properties of experimental soil were: Clay 6%, Silt 49%, Sand 45%, pH 6.0 ± 0.1 , TOC $3.60 \pm 0.00\%$, CEC $149 \pm 5.90 \text{ mmol kg}^{-1}$, P_{tot} $0.02 \pm 0.00\%$, K_{tot} $0.25 \pm 0.00\%$, Mg_{tot} $0.12 \pm 0.00\%$, Ca_{tot} $0.14 \pm 0.01\%$, S_{tot} $0.03 \pm 0.00\%$, Fe_{tot} $11.76 \pm 0.62\%$, Mn_{tot} $0.14 \pm 0.00\%$, Zn_{tot} $7595.65 \pm 194.76 \text{ ppm}$, Cd_{tot} $80.82 \pm 4.58 \text{ ppm}$, Cu_{tot} $62.51 \pm 6.45 \text{ ppm}$, Pb_{tot} $4346.13 \pm 140.08 \text{ ppm}$, Ni_{tot} $5.61 \pm 0.25 \text{ ppm}$, As_{tot} $177.54 \pm 5.21 \text{ ppm}$.

2.2. Amendments and greenhouse experiment

Three materials, namely BC derived from wood chips gasification (150 kW/h gas and 300 kW/h heat production) at the temperature range 700–900 °C, WA which was collected from a fluidized bed reactor (15 MWt) for wood chips burning at a commercial biomass power plant, and commercial potassium HS Lignohumate (Amagro, Czech Republic) produced by alkaline extraction from lignin were used as soil amendments in this study. The elemental composition of BC, WA, and HS is shown in Table 1.

Amendments were applied to soil at different doses in order to achieve the equal pH values (from initial pH 6.0 in control treatment):

- pH~6.5 with the addition of the BC 0.5%, or HS 0.5%, or WA 2.8%;
- pH~7.0 with the addition of the BC 5%, or HS 1%, or WA 6%.

It should be noted, that each treatment induced changes in the initial total organic carbon concentration (TOC) in soil. The addition values for TOC with each treatment were: 4.4 g kg^{-1} with BC 0.5%, 1.7 g kg^{-1} with HS 0.5%, 2.4 g kg^{-1} with WA 2.8%, 44.1 g kg^{-1} with BC 5%, 3.3 g kg^{-1} with HS 1%, 5.1 g kg^{-1} with WA 6%.

Each portion of a 200 g air-dried soil sample was mixed with a dried amendment and placed into 0.5 L plastic pots (10 cm diameter and 15 cm height). The moisture content was maintained at 60% of the respective maximum water holding capacity (WHC) and kept constant by watering up to original weight every third day, which resulted in a maximum water loss of 5%. Pots were incubated uncovered at a temperature of 21/18 °C (day/night) in the natural light regime for 60 days. The non-amended (NA) and amended soils were collected after 30 and 60 days of incubation. At the end-point, each sample was properly mixed and divided into two parts: one part was stored at 4 °C for enzymes, and another part was dried at 105 °C for chemical analyses.

2.3. Chemical properties

The pH values of the non-amended and amended soils were measured in 0.01M CaCl_2 at a ratio of 1:2 (w/v) using WTW pH 340i meter with glass, ion-selective electrode (WTW, Weilheim, Germany).

The mobility of TEs (Cu, Zn, As, Cd, and Pb) was determined by means of sequential extraction procedure according to Száková et al. (1999):

Table 1
Chemical characteristic of amendments.

Characteristics	Amendment		
	BC	WA	HS
pH	8.9	11.2	9.0
N,%	0.44	0.00	0.25
C,%	88.20	8.50	33.47
H,%	0.82	0.16	3.73
S,%	0.19	0.33	4.84
O,%	6.49	4.79	17.72
Ash, total (%):	3.86	86.22	40.0
Na, %	<0.001	0.04	22.5
K,%	0.59	2.93	3.61
Mg, %	0.02	1.73	0.04
Al, %	0.04	2.84	0.01
Si, %	1.41	20.58	0.08
P, %	0.05	1.01	0.06
S, %	0.03	1.97	3.86
Ca, %	0.98	11.84	0.93
As, %	0.002	<0.001	<0.001
Zn,%	0.01	0.12	0.04
Cd, %	0.02	<0.001	0.01
Pb, %	<0.001	0.16	0.06
Cu, %	0.06	0.01	0.01
Other, %	0.64	42.99	8.79

All values are given on moist-free basis.

- i) water-soluble fraction - 1 g of soil samples was mixed with 10 ml of demi-water and was shaken for 2 h at room temperature, the extract was separated from the solid by centrifugation (8000 g × 10 min), decanted into a polyethylene bottle and stored at 4 °C;
- ii) exchangeable fraction - 40 ml of 0.11 M acetic acid (pH 2.0) was added to the residue and shaken for 16 h at room temperature, followed by the same procedure described for step i.

The TE's concentrations in the supernatants were determined by an inductively coupled plasma – optical emission spectrometer (ICP-OES, Agilent 720, Agilent Technologies Inc., USA). All plastic and glassware were acid-soaked overnight (5% HNO₃) and rinsed with distilled water before use.

The changes in the mobile species of trace elements in soil were evaluated using a modified partition index (PI) calculated according to Han et al. (2003):

$$PI_i = \frac{C_i(\text{water-soluble}) + C_i(\text{exchangeable})}{C_i(\text{total})} * 100\%$$

where *i* is the selected element (Cu, Zn, As, Cd, or Pb). *C_i* (total) – the initial pseudo-total concentration of TE in soil.

2.4. Soil enzyme activity

Dehydrogenase (EC 1.1) activity was evaluated according to Thalmann (1968) and (was) expressed as μg TPF g⁻¹ soil 16 h⁻¹. The activities of acid phosphatase (EC 3.1.3.2) in the soil samples were assayed as outlined by Eivazi and Tabatabai (1977) and were expressed as μg pNP released g⁻¹ dry soil h⁻¹. β-glucosidase activity (EC 3.2.1.21) was determined according to the procedure described by Dick et al. (1996). The Results are expressed as μg pNP released g⁻¹ h⁻¹ dry soil. The Urease activity (EC 3.5.1.5) was evaluated according to the method described by Klose and Tabatabai (2000) and (was) expressed as μg NH₄⁺ g⁻¹ soil 24 h⁻¹. The hydrolysis of fluorescein diacetate (EC 3.2.1.21) was determined by a modified procedure of Inbar et al. (1991) and expressed as μg FDA g⁻¹ h⁻¹ of soil. For all enzyme activities, the assays were performed in triplicate and were corrected for a blank.

Soil microbial activities were combined in to the weighted mean index (WMean) described by Lessard et al. (2014):

$$WMean = \sum_{i=1}^n w_i * y_i$$

where *y_i* - is the activity of *i*-enzyme, *n* - is the total number of soil enzymes, and *w_i* - is the 'weight' of each soil enzyme that was calculated as:

$$w_i = \frac{v_i}{\sum_{i=1}^n v_i}$$

being *v_i* the eigenvector for each soil enzyme activity associated with the first or second (depends on the data) principal component obtained from a PCA.

This index is regarded to be a reliable tool to integrate information from variables that possess different units that are featured by a range of variation indicator in a diversity of contaminated soils (Lessard et al., 2014; Sanchez-Hernandez et al., 2017). Compared with many other enzyme-based indices, WMean index is calculated according to the PCA-results and summarizes the 'weighted' values, so the relative importance for each response is evaluated objectively.

2.5. Earthworms bioassay

The *Eisenia foetida* mortality bioassay was carried out according to the Organisation for Economic Co-operation and Development (OECD) procedure (OECD 207/222). A homogeneous group of earthworms was acclimated for 2 weeks in 1 box with artificial soil (10% peat, 20% clay and 70% quartz sand, pH 6–7 adjusted with calcium carbonate) at 18 ± 1 °C, and 16:8 h light/dark regime. The earthworms were cleaned and kept in darkness for 24 h before use. After this acclimation every ten *E. foetida* earthworms (each organism weighing 0.2–0.5 g) were placed in an aluminium box containing 200 g of dry soil + amendment (70% WHC moisture) in four replicates. The container was covered by polyethylene material with dots in order to prevent evaporation. Approximately 2.5 g of food (oatmeal) with water was spread on the soil surface of each container every week. After 30 days of laboratory experiment, the earthworms were removed from the soil. The earthworms were cleaned from soil particles and the following variables were determined: survival rate (SR), individual biomass changes (IB). The number of cocoons was also counted after 60 days.

The survival rate (SR) was calculated as follows:

$$SR = \frac{N_{adi}}{N_{ad_{oeed}}} * 100\%$$

where *i* – data for the samples (NA, or treatment), OECD – data for an artificial soil control, *N_{ad}* – number of living adult earthworms after 30 days of exposure.

Individual biomass changes (%) were calculated as follows:

$$IB = \frac{IB_i}{IB_{oeed}} * 100\%$$

where *IB_i* – individual earthworms biomass in samples (NA, or treatment), *IB_{OECD}* – individual earthworms biomass in artificial soil control.

2.6. Statistical analyses

All treatments were conducted in 4 independent replicates. The analyses were iterated in 3 technical replicates for each sample and the mean value of these 3 was further used as a result of the measurement for each sample in further interpretation and statistical evaluation.

To test the effects of the experimental factors (type of amendment, dose of amendment, sampling time) in the variables analysed, a tree-way analysis of variance (ANOVA) with interactions was performed. In the case of significant F-tests, differences between group means were assessed by the Fisher's *post hoc* least significant difference test (LSD) with the significance level at $p < 0.05$. The variance homogeneity was verified by a graphical analysis of the residuals and no transformation was necessary. The correlation between characteristics was calculated using Pearson's rank correlation with the level of significance established at $p < 0.05$ by using Statistica 10.0 (StatSoft, Tulsa, OK). All graphs were prepared using SigmaPlot 12.5 (Systat, San Jose, CA).

Principal components analysis (PCA) was used for WMean index calculation and the component extraction was made by the covariance (n) matrix using XLSTAT-Ecology software.

3. Results

3.1. Amendments effect on soil chemical properties

In this study, the efficiency of the TE's immobilization was estimated on the basis of water-soluble and exchangeable species, and significant effects of BC, WA, and HS treatments on soil characteristics were revealed (Table 2). All treatments induced alkaline effect and changed As, Cu, Zn, Cd and Pb mobility (predominately in

water-extractable species than in exchangeable ones). Moreover, the effects strongly depended on the type of amendment and the studied element (the results of the Factorial ANOVA test are presented in Supplementary Table A, ST A).

The $\text{pH}_{\text{CaCl}_2}$ values for all samples tested ranged from 6.47 to 7.18 in comparison with the non-amended control (pH 6.04–6.12) (Table 2). The BC 5%-treated soil exhibited a significant water-soluble Cu-immobilization effect in contrast with other amendments (Table 2; ST A), and in general, higher doses of amendments had more influence compared to lower doses at 30- days. The significant reduction in water-soluble and acid-extractable Cu concentration was found after exposure period of 30-days for higher doses of amendments.

Overall, BC demonstrated a weak influence on Zn-concentration (only slightly or insignificantly influenced). The only difference from NA was found in Zn water-soluble species after 60 days of exposure. HS seemed to have a prolonged effect on Zn immobilization as the significant reduction was found only after 60 days. The most intensive immobilization was determined in the case of WA. Generally, Zn concentration significantly and negatively correlated with the initial TOC and $\text{pH}_{\text{CaCl}_2}$ values (Table 3).

Arsenic available concentration in soil was influenced by several factors: the type of amendment, treatment dose, time and pH-factor. The BC-treated soil was characterized by the lowest concentration of As ($0.26\text{--}0.30\text{ mg kg}^{-1}$), while HS and WA had a weaker impact on As compare to BC (Table 2; ST A). The immobilization effect also increased with the increasing doses of amendments and time of exposure, as well as the increased $\text{pH}_{\text{CaCl}_2}$ values which correlated with a decreased available As concentration in soil (Table 3).

All treatments significantly affected mobile Pb concentration in soil. The concentration of water-soluble Pb was the lowest after BC

Table 2

Leachability of trace elements from the polluted soil in the presence of amendments. The data represent Mean (mg kg^{-1}) \pm SD ($n = 4$). Different letters denote significant differences at the 0.05 confidence level (Fisher's LSD) between treatments at the same exposure time.

Amendment	$\text{pH}(\text{CaCl}_2)$		As			
	30d	60d	30 d		60 d	
			H ₂ O	CH ₃ COOH	H ₂ O	CH ₃ COOH
NA (control)	6.11 \pm 0.05 a	6.10 \pm 0.03 a	0.45 \pm 0.06 a	1.61 \pm 0.16a	0.34 \pm 0.04 a	1.46 \pm 0.06 a
BC 0.5	6.40 \pm 0.10 b	6.41 \pm 0.05 b	0.29 \pm 0.01 b	1.30 \pm 0.19 b	0.27 \pm 0.03 b	1.21 \pm 0.19 b
BC 5	6.80 \pm 0.04 c	6.90 \pm 0.05 c	0.30 \pm 0.03 b	1.41 \pm 0.15 ab	0.26 \pm 0.04 b	1.25 \pm 0.11 b
WA 2.8	6.50 \pm 0.05 b	6.53 \pm 0.13 b	0.45 \pm 0.06 a	1.44 \pm 0.22 ab	0.35 \pm 0.06 a	1.31 \pm 0.34 ab
WA 6.0	7.08 \pm 0.03 c	7.15 \pm 0.05 c	0.29 \pm 0.01 b	1.34 \pm 0.69 b	0.29 \pm 0.01 ab	1.16 \pm 0.13 b
HS 0.5	6.60 \pm 0.01 b	6.55 \pm 0.06 b	0.37 \pm 0.06 ab	1.45 \pm 0.23 ab	0.28 \pm 0.02b	1.11 \pm 0.23 b
HS 1	7.05 \pm 0.06 c	7.10 \pm 0.03 c	0.29 \pm 0.01 b	1.53 \pm 0.06 ab	0.28 \pm 0.00b	1.21 \pm 0.13 b

Amendment	30 d		60 d		30 d		60 d		
	H ₂ O	CH ₃ COOH	H ₂ O	CH ₃ COOH	H ₂ O	CH ₃ COOH	H ₂ O	CH ₃ COOH	
Zn				Pb					
NA (control)	8.04 \pm 2.16 ab	1785.7 \pm 161.6 a	7.01 \pm 0.80 d	1511.0 \pm 65.4 b	0.90 \pm 0.50 c	95.1 \pm 9.1 a	0.78 \pm 0.11 d	86.0 \pm 4.7 a	
BC 0.5	5.72 \pm 0.82 ab	1852.6 \pm 223.6 a	4.58 \pm 0.38 a	1422.9 \pm 160.9 ab	0.31 \pm 0.11 ab	92.4 \pm 19.3 a	0.38 \pm 0.02 c	82.3 \pm 4.4 a	
BC 5	4.40 \pm 3.09 acd	1628.5 \pm 80.5 a	3.01 \pm 1.32 c	1379.5 \pm 158.1 ab	0.20 \pm 0.01 a	80.3 \pm 9.1 a	0.18 \pm 0.03 a	82.3 \pm 2.4 a	
WA 2.8	2.90 \pm 1.44 cd	1418.1 \pm 238.7 a	1.78 \pm 0.06 bc	984.7 \pm 78.4 d	0.49 \pm 0.00 bc	67.8 \pm 18.4 a	0.40 \pm 0.01 c	55.4 \pm 6.0 b	
WA 6.0	1.62 \pm 0.21 c	1576.9 \pm 242.2 a	1.49 \pm 0.10 b	838.1 \pm 56.2 c	0.33 \pm 0.15 ab	71.8 \pm 24.8 a	0.29 \pm 0.02 b	58.3 \pm 1.6 b	
HS 0.5	6.32 \pm 1.47 ab	1551.6 \pm 495.4 a	5.81 \pm 0.60 ad	1380.8 \pm 129.1 ab	0.25 \pm 0.03 a	71.6 \pm 32.2 a	0.27 \pm 0.02 b	64.8 \pm 2.8 c	
HS 1	7.97 \pm 2.96 b	1623.0 \pm 108.8 a	5.03 \pm 1.04 a	1277.1 \pm 50.6 a	0.22 \pm 0.05 a	80.9 \pm 17.1 a	0.19 \pm 0.00 a	65.1 \pm 3.1 c	

Amendment	Cd		Cu	
	H ₂ O	CH ₃ COOH	H ₂ O	CH ₃ COOH
NA (control)	0.09 \pm 0.02 a	14.8 \pm 2.9 a	0.05 \pm 0.00 a	15.2 \pm 0.3 a
BC 0.5	0.07 \pm 0.01 a	16.0 \pm 1.4 a	0.05 \pm 0.01 a	7.6 \pm 1.1 b
BC 5	0.06 \pm 0.03 ab	13.5 \pm 0.8 a	0.04 \pm 0.01 a	7.6 \pm 1.2 b
WA 2.8	0.04 \pm 0.00 b	14.8 \pm 1.8 a	0.05 \pm 0.00 a	8.0 \pm 0.6 b
WA 6.0	0.06 \pm 0.03 ab	12.1 \pm 2.0 a	0.05 \pm 0.01 a	7.4 \pm 0.4 b
HS 0.5	0.08 \pm 0.02 a	14.3 \pm 2.7 a	0.05 \pm 0.01 a	7.2 \pm 0.2 b
HS 1	0.08 \pm 0.01 a	13.3 \pm 4.8 a	0.04 \pm 0.00 a	8.4 \pm 1.1 b

Abbreviation H₂O – the water-soluble species; CH₃COOH – the exchangeable species of the elements.

Table 3
Pearson correlations matrix for soil variables, soil enzymes activity, and earthworms bioassay endpoints.

Variables	TOC	pH(CaCl ₂)	Soil enzyme activity					Earthworms			
			Dehydrogenase	Urease	Acid phosphatase	FDA	β-glucosidase	Survival rate	Individual biomass	Cocoons	
TOC	1	0.75	0.42	0.11	-0.55	0.50	-0.15	-0.37	-0.23	-0.21	
pH(CaCl ₂)	0.75	1	0.36	0.09	-0.17	0.36	-0.17	0.03	0.19	0.27	
H ₂ O	As	-0.47	-0.35	-0.49	-0.44	0.04	-0.48	-0.33	0.28	0.29	0.00
	Pb	-0.66	-0.68	-0.25	-0.06	0.26	-0.61	-0.08	0.03	-0.09	-0.29
	Zn	-0.56	-0.67	0.15	0.32	0.55	-0.17	0.33	-0.18	-0.37	-0.30
	Cu	-0.61	-0.68	-0.23	-0.02	0.28	-0.56	-0.11	-0.08	-0.20	-0.36
	Cd	-0.57	-0.62	0.00	0.21	0.38	-0.20	0.13	-0.14	-0.34	-0.21
CH ₃ COOH	As	0.10	-0.07	0.01	-0.08	-0.13	-0.16	-0.03	-0.23	-0.13	-0.39
	Pb	-0.40	-0.40	0.14	0.17	0.36	0.02	0.32	-0.17	-0.16	-0.22
	Zn	-0.42	-0.47	-0.01	0.15	0.26	-0.13	0.18	-0.07	-0.15	-0.22
	Cu	-0.52	-0.54	0.02	0.26	0.34	-0.29	0.11	-0.03	-0.32	-0.22
	Cd	-0.39	-0.24	0.08	0.18	0.34	-0.02	0.21	0.08	-0.06	0.05

The values in bold mark significant correlation at the 0.05 level.

and HS amendments, while WA predominantly affected the exchangeable Pb species (Table 2). Increasing the doses of amendments had marked effects on Pb-concentration (ST A), and the pH_{CaCl2} and TOC concentration showed a negative correlation with Pb concentration in soil (Table 3).

Cd available concentration in soil had a slight trend to decrease water-soluble and exchangeable species with a presence of BC 5%, WA 6%, and HS 1% (Table 2). Overall, Cd immobilization was stronger with increasing the doses of amendments and time of exposure (ST A). WA had more impact than BC and HS.

A partition index indicates the percentage of trace element presented in the water-soluble and exchangeable species versus the pseudo-total concentration. The decrease in the partition index demonstrates that the chemical species of the element are changed to less available form (Fig. 1). All the amendments were demonstrated to be able to immobilize trace elements but with different extent. BC treatments influenced preferably Zn and Cu mobility

(Fig. 1A, E). WA proved to be more effective in reducing Zn and Cu in both treatment doses, and decreased Pb mobility with higher application dose (WA 6) (-25.7%) (Fig. 1 A, B, E). HS decreased Cu and Zn concentration and a slight decrease was found also for Pb (Fig. 1 A, B, E). It should be noted that we observed a trend to decrease As mobility only with 0.5% HS treatment (Fig. 1 C).

3.2. Effects in the enzymes activity

Dehydrogenase activities tend to increase with the elevated doses of all amendments (Fig. 2A; ST B) and according to the elevated level of initial TOC values (Table 3), but the absolute values of DHA activity were decreased over time (60 days). BC and HS-treated soils supported dehydrogenase activity, while WA had slightly negative or no effect on dehydrogenase. Generally, the dehydrogenase activity increased by 45–80% after 30 days, and 57–240% after 60-days in BC and HS treatments and no effect was

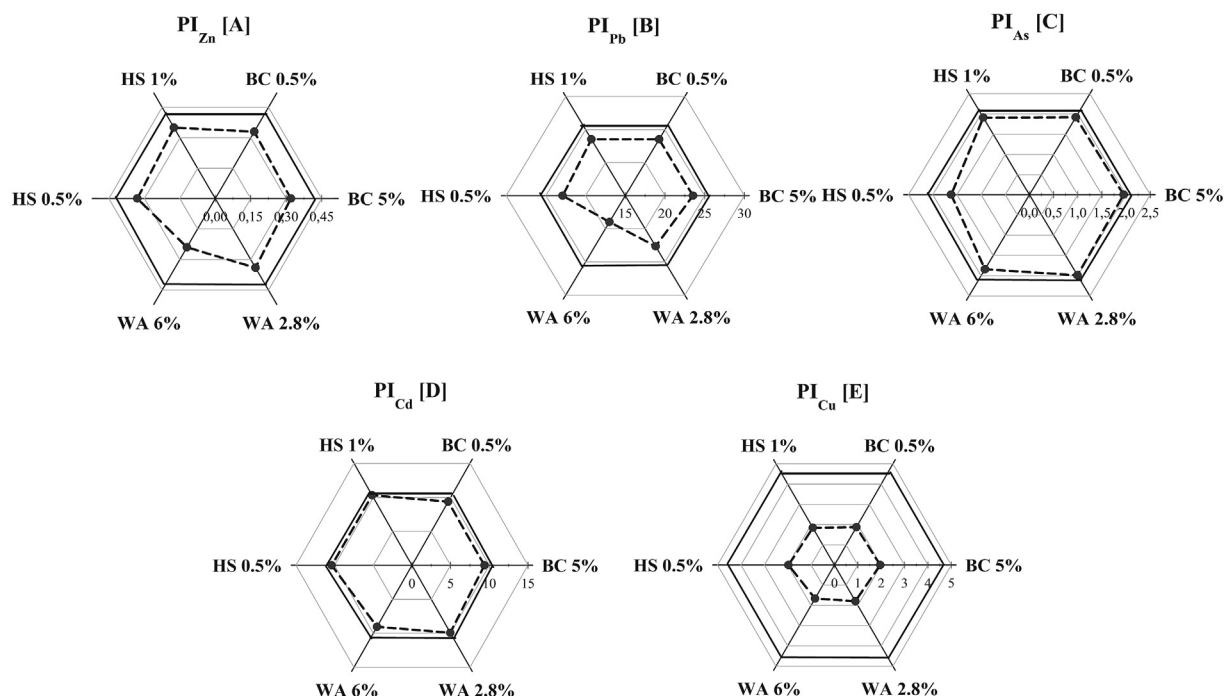


Fig. 1. Changes in the mobility of trace elements in soil according to the Zn (A), Pb (B), As (C), Cd (D), Cu (E) Partition index with a presence of different amendments at the end of experiment. The partition index for NA shows in black solid line, for each element – in medium dash line.

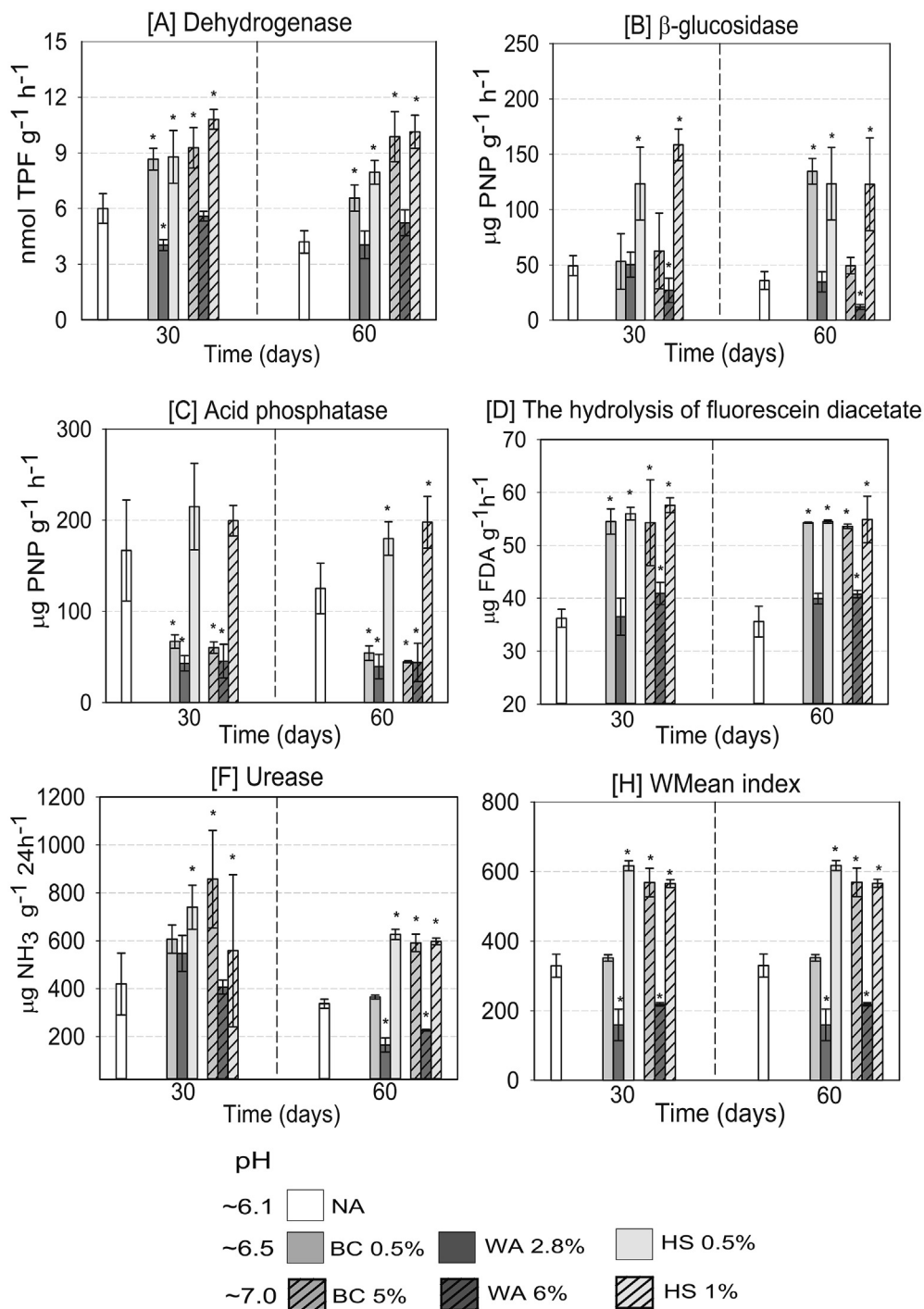


Fig. 2. Enzyme activities (A–F) and summarized Weighted Mean index (H) in soil with varying doses of amendments (Mean \pm SD, $n = 4$) after 30, and 60-days of exposure. The * highlights significant differences in treatments compared to control (NA) (Fisher's LSD; $p < 0.05$).

found in case of WA after 60 days.

BC and HS additions have also a positive impact on the β -glucosidase activity (Fig. 2B; ST B). The effect of HS was well expressed in both doses at both incubation periods while BC showed significant improvement of β -glucosidase only in the lower dose after 60 days. Generally, the influence of amendments was more expressed in 0.5% BC and 0.5% HS treatments than in higher doses. The activity of β -glucosidase also decreased over time through during the experiment. WA demonstrated a trend to decrease the β -glucosidase activity. Moreover, a higher application

dose of WA led to a significantly inhibition of β -glucosidase (lowest values $4.21 \pm 0.21 \mu\text{g pNP g}^{-1} \text{ dry soil h}^{-1}$ after 60 days).

The quantification of acid phosphatases activity revealed a significant promotion by HS-treatments at 60 days (Fig. 2C; ST B), while the other types of amendments had significant inhibitory effects when compared with non-amended soil. The acid phosphatases activity slightly decreased with increasing the exposure time, at HS treatments (only at 60 days).

The hydrolysis of fluorescein diacetate in soil treated with carbonaceous amendments (BC and HS) markedly improved by

both application doses at both sampling periods, while the positive effect of WA was determined only in case of higher application dose (Fig. 2D; ST B).

The urease soil activity dramatically increased with BC and HS additions (Fig. 2F; ST B), and the effect was intensified with an elevated application dose. WA-treated soils were characterized by no effect (30 days) or significant decrease (60 days) in activity of these enzymes. During the experiment, the values of urease activities significantly decreased in all treatments over time.

Finally, according to the calculated Weighted Mean index (WMean), BC and HS amendments triggered a marked improvement of soil microbial conditions, while WA treatments had a trend to depress microbial activity despite the highest trace element immobilization efficiency (Fig. 2E).

3.3. Earthworms responses

The earthworm mass decreased by 40% in NA treatment after 30-days of exposure in comparison with OECD artificial soil. The average number of cocoons per 10 earthworms in NA soil was 21 as compared with 53 in the OECD soil, indicating a considerable effect of adverse soil conditions on cocoon production (Fig. 3C). Similarly, a lower dose of BC and HS resulted in 40–50% body mass decrease (no significant difference from NA) (Fig. 3A). Surprisingly, applying a higher dose of BC 5% led to annihilated of 100% earthworms with no cocoons detectable after 60 days (Fig. 3C). Contrarily, the application of both WA doses resulted in a significant increase of earthworm's body mass (Fig. 3A), 100% survival rate (Fig. 3B) and the higher dose of WA also led to slightly higher number of cocoons compared to OECD control (Fig. 3C).

4. Discussion

4.1. Mobility of trace elements

Many types of amendments have been reported as effective

supplements to remediate the contaminated sites. The multi-contaminated soil near the Trhové Dušníky was also previously studied with regards to the use of different materials for soil recovery. For example, lime was proved to reduce Cd and Zn mobility, yet turned to be ineffective to reduce Pb and As mobility (Vondráčková et al., 2013). Digestate and fly ash were observed as successful amendments for Cd, Zn and Pb immobilization (García-Sánchez et al., 2015). The WA, BC, and HS also have been effective to reduce Cu, Zn and Cd plant-available concentration in soil (Pukalchik et al., 2017). In this study, we focused on the effects of BC, WA, and HS to the bind forms of Cu, Zn, As, Cd and Pb which may equilibrate with the aqueous phase and acetic acid, as those are considered to be rapidly bioavailable (Seguin et al., 2004; Kabata-Pendias, 2011). The soil water extraction is suitable for evaluating metal concentrations at a pseudo equilibrium in the soil solution (Meers et al., 2007).

The results demonstrated different potential of BC, WA, and HS to short-term reduction of water-soluble and exchangeable forms of TEs in soil. It was observed that BC (0.5 and 5%) evoked marked decrease in Cu, and had a tendency to decrease Zn available concentrations; WA-treatments (2.8 and 6%) had strong influence on the reduction of Zn, Pb and Cu mobile soil concentration, while HS (0.5 and 1%) primary changed Cu soluble concentration in polluted soils.

The selectivity of these amendments to reduce mobile fractions of TEs were largely expected. More important findings demonstrate different mechanisms of remediation efficiency of three tested amendments. The BC addition affected Cu and Zn concentration, while a decrease in other elements was negligible. It may be linked with formation of metal hydroxides, oxides, carbonates, and phosphate precipitates and with the activation of surfaces caused by the increase in pH (Uchimiya et al., 2011). Han et al. (2017) investigated the competitive adsorption of Pb and Cd by biochars produced from 12 sources, indicating that the Pb adsorption process was inhibited in the mixed solutions with Cd. This competition also was obtained in other binary systems such as Cu and Zn (Chen

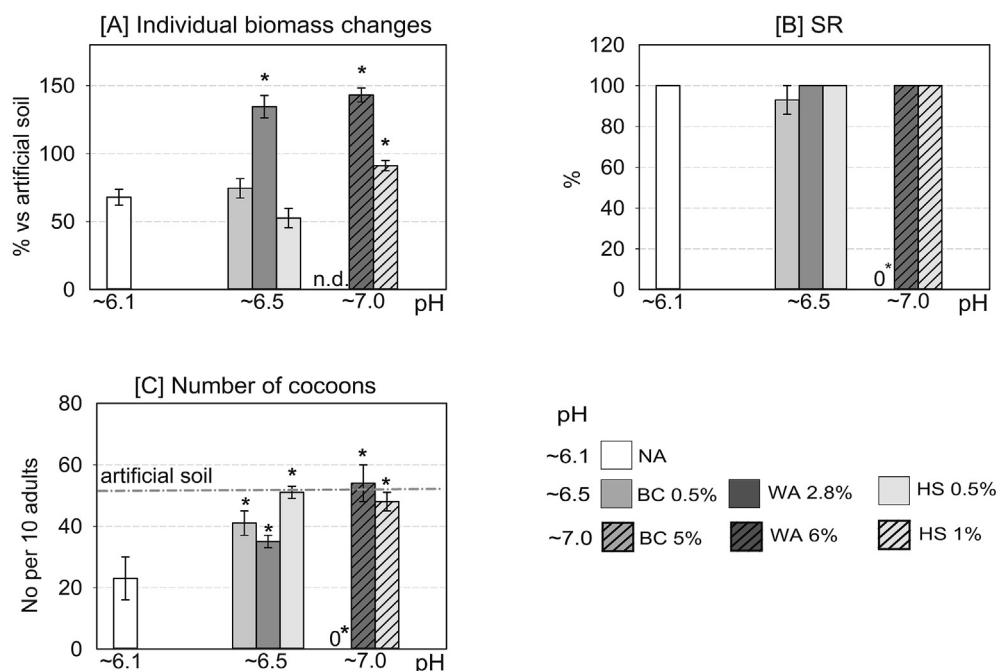


Fig. 3. Individual biomass changes (A), survival rate (B), and number of cocoons production (C) of *Eisenia foetida* after exposure in soils with different amendments (Mean \pm SE, n = 4). The * highlights significant differences in treatments compared to control (NA) (Fisher's LSD; $p < 0.05$); n. d. – data not detected.

et al., 2011), and multi-metals system (Park et al., 2015). Those results may imply that metals could be adsorbed by the same sites and functional groups, while competing with other metals to produce reciprocal inhibition, and there are different mechanisms and bindings for various metals (Chen et al., 2011).

HS primary changed Cu concentration in contaminated soil. As all heterogeneous macromolecules, HS contains multifunctional groups, among them the carboxylic and phenolic–OH, which are known to be the most important in forming the negative charges of humic substances for metal complexation (Wang et al., 2010; Shan et al., 2015). The dissociation constant (pKa) for COOH are 3.0 and 10.0 for phenolic–OH (Tan, 2014), implying that the phenolic–OH would be mainly protonated under this liming condition (pH 6.5–7.0), whereas COOH would be deprotonated to an effective complex with positively charged metals. It also has been reported that the sorption of Cu ions on HS is mainly through the formation of five-membered chelate rings via ion exchange and surface complexation, respectively (Karlsson et al., 2006).

Finally, adding WA was more effective than BC and HS treatments in removing Cu, Pb and Zn from the available pool of contaminated soils. This effect may be attributed to high soluble concentrations of carbonates and phosphates in wood ash, and the main mechanism for metals sorption was suggested as the formation of precipitates, such as $ZnCO_3$, $Zn_5(CO_3)_2(OH)_6$ (Voegelin et al., 2005), $Pb_5(PO_4)_3OH$ (Cao et al., 2011), and $Cu(OH)^+$ (Chirenje et al., 2006).

According to Masscheleyn et al. (1991), the arsenic is normally enhancing solubility in soil with alkaline pH. In particular, the soil liming above 6.5 induced changes in As mineral saturation from As^0 , H_3AsO_3 to $HAsO_4^{1-}$. In line with this, the addition of alkaline amendments should increase the As water-soluble and exchangeable species in soil. However, our data revealed quite the opposite effect: partition index for As did not significantly change, or slightly decreased at the background of amendments (Fig. 1). Thus, we can conclude that all tested amendments successfully neutralised the As mobility in soil. Similar effect was previously reported by Vandecasteele et al. (2002), and immobilization of As in contaminated soils with a presence of fly ash was linked with the formation of $Ca_3(AsO_4)_2$ and $CaHAsO_3$.

The decrease of mobile TEs concentration may be also attributed to the increasing soil pH_{CaCl_2} value, significant negative correlation at $p < 0.05$ between water-soluble As, Pb, Zn, Cu, Cd, exchangeable Pb, Zn, Cu, Cd were observed (Table 3). At the same time, the applied amendments showed a considerable increase of TOC in the soil, so this effect may be linked with changes in carbon concentration.

4.2. Soil enzyme activities

The hydrolytic enzymes determined in our study provide an index of the potential to mineralize C (β -glucosidase, dehydrogenase), and the nutrients N (urease), P (phosphatase), which can be used as early indication of changes in soil quality (Ndiaye et al., 2000; Tejada et al., 2006). Fluorescein diacetate hydrolysis activities are related to the overall microbial activity, and have been investigated to determine the amounts of active fungi and bacteria, and hence may considerably affect the soil organic matter dynamics (Lundgren, 1981; Aseri and Tarafdar, 2006).

This study has shown that short-term WA exposure caused a significant decrease in the activity of dehydrogenase, acid phosphatase and β -glucosidase, as well as in soil microbial activity as indicated by the reduced FDA activities. This fact is in line with the observed effect of WA on the WMean index (Fig. 2E). Despite the data of WA impact on soil enzymatic activity are scarce, our results are consistent with those of other studies (Perucci et al., 2006;

Björk et al., 2010). Perucci et al. (2008) showed that the inhibitory effect of wood ash on soil enzymatic activities lasts shorter (up to 12 months) and that its application does not result in long-term changes of enzymatic activities in field conditions. A recent study also demonstrated that the WA can decrease the ratio of fungi to bacteria in the soil microbial community (Noyce et al., 2016).

Application of BC has been reported to influence C and N mineralization in soil, and could increase the activity of specific enzymes related to C, N utilization (Bailey et al., 2011; Yang et al., 2016), and our data supported this fact. BC positively affected urease, β -glucosidase activities compared to non-amended soil. The β -glucosidase catalyses the last step of cellulose hydrolysis and release of glucose as energy source for the microorganisms. Its potential activity is associated with carbon substrate availability, so our data suggested that BC and HS might affect the soil community probably through increasing initial TOC rather than reducing the TE's mobility. The acid phosphatase activity decreased significantly with the application of BC; similar effects were previously observed with 5% bamboo BC (Yang et al., 2016), and BC from *Parthenium hysterophorus* (Kumar et al., 2013). Dehydrogenase and fluorescein diacetate hydrolysis activities both were significantly higher in BC soil than in NA soil. Possibly, it can be explained by the stimulation of a specialized subset of the microbial community by the biochar or growth of biomass in response to initially labile C (Bailey et al., 2011).

The results of the experiments revealed a stimulating effect of HS on the enzymes activity, which is not surprising if we consider its positive influence on microbial populations. It is noteworthy, that HS treatments differed from WA and BC treatments in promoting the acid phosphatase activities. A higher acid phosphatase activity in samples with HS are opposite to the fact that phosphomonoesterase is an enzyme which is very sensitive to changes in soil pH (Dick et al., 2000), and the optimum pH for the activity of acid phosphatase ranges from 4.0 to 6.5 (Wittmann et al., 2004). It has to be noted that in our experiment, we found no significant differences between treatments in activity of alkaline phosphatase and therefore, these data are not shown. According to Nannipieri et al. (2011), a linear relationship is commonly observed between the activity of acid phosphatases and the amount of phosphorus in soil solution, which could be provided from humic products. Tikhonov et al. (2010) observed that some fractions of HS are labile and can provide a ready-to-use substrate for microbial populations. A similar trend with fluorescein diacetate hydrolysis activities stimulation was previously pointed with HS from industrially mined raised bog peat in sandy loam soil spiked with a complex contamination (Muter et al., 2015).

Generally, the enzyme activity correlated with the TOC in soil (Ekenler and Tabatabai, 2003; Darby et al., 2006). We found positive and significant correlations of the initial added amount of TOC with dehydrogenase activity, and fluorescein diacetate hydrolysis activities (Table 3), however, there was no relationship with β -glucosidase, urease and acid phosphatase. This fact may indicate that only extracellular enzymes are fixed in the soil matrix by interacting with organic carbon. Moreover, the fluorescein diacetate hydrolysis activities was the only enzyme activity, which negatively correlated with all the measured water-soluble As, Cu and Pb concentration, while dehydrogenase and urease was negatively affected only by water-soluble As (Table 3). These results are opposite to Pan and Yu's conclusions (2011), who reported that the metals such as Cu, Cd and Pb induced marked inhibition of dehydrogenase activity. Our results suggested that fluorescein diacetate hydrolysis activities were the best representatives to be used as a biological indicator for soil recovery as compared to all other enzymes tested. This data also confirms a direct inhibitory effect of the most labile (water-soluble) species of metals on the overall microbial activity.

Applying the integrated enzymes-based index provides comprehensive information about the biological effects of contamination and may therefore serve as a useful tool for environmental managers (Paz-Ferreiro and Fu, 2016). Among them, the Wmean index has been satisfactorily validated in the assessment of metal-contaminated soils (Lessard et al., 2014) and pesticide-contaminated soils (Sanchez-Hernandez et al., 2017). To the best of our knowledge, this study is the first to use WMean index to assess soil health status based on soil microbial activities during the remediation. The highest improvements were obtained both in BC and HS treatments, while WA depressed the soil microbial community (Fig. 2E).

4.3. Earthworms survival

Earthworms are known for their sensitivity to toxic chemicals present in contaminated soils and hence have been widely used as indicator organisms for ecotoxicity studies. They can pose a risk to higher trophic levels that feed upon them (Suthar and Singh, 2009). However, the earthworm survival in such stress conditions - like contaminated soils - depends upon several physicochemical factors such as soil texture, pH_{CaCl2} of the medium, organic matter, nature and extent of the clay minerals (Maity et al., 2008). In our study, the TE's stress was probably to cause a significant body weight reduction in non-amended soil whereas WA (2.8% and 6%) and HS 1% showed the opposite as compared to NA soil. The decrease in body weight observed in control (NA) earthworms may account for the lower availability of food resources as the TOC was much lower than in amended soil. We also observed the 100% mortality of adult earthworms in the BC 5% treatment during the first 5 days of the experiment, whereas no acute mortality occurred in the control soil and other treatments. Toxicity of BC for earthworms was previously detected by Li et al. (2011), soil amended with 10% and 20% apple wood BC induced significant reduction in worm's body-mass during 28d test due to changes in soil water holding capacity. Liesch et al. (2010) observed higher earthworm death rate and weight loss in 67.5 t/ha poultry litter BC amended soil, due to the rapid increment in soil pH or excessive salinization. Earthworms in arable soil treated with a pruce chip BC (30 t/ha) tended to avoidans biochar after 2-week of exposure due to a decline in soil water potential (Tammeorg et al., 2014). Similarly, Malev et al. (2016) observed that BC application at the rate of 100 t/ha could cause damage to earthworm, with survival rates decreasing to 78% in clay soil and 64% in sandy soil.

4.4. Integrative approach

In our study, the mixtures of TE's in multi-contaminated soils interacted with contrasting amendments and induced the magnitude of effects in chemical, biological and ecotoxicity properties of soil. The generalized data for biological and ecotoxicity responses in presence of WA, HS, and BC from this study and the previous one (Pukalchik et al., 2017) are shown at Table 4.

We could suggest that each type and dose of amendment has unique advantages and disadvantage, making it very hard to choose the 'master key' - the universal variant that would miraculously solve a plethora of problems facing the soil protection today. We detected the adverse effect of WA, BC and HS on soil biological and ecotoxicity properties. BC 5% were harmful for earthworms, while WA application supported earthworms reproduction and biomass but depressed enzymes activity. Overall, the WA 6% and BC 5% had the greatest number of negative effects among all treatments, while WA 2.8%, BC 0.5%, HS 0.5% and HS 1% promoted good (or normal) microbial performance and created suitable conditions to the organisms assayed (at least has no harmful effect). Our data proved an

Table 4

Summary table of effects observed in each biological parameter in soils with a presence of amendments.

Parameter	BC		WA		HS	
	0.5%	5%	2.8%	6%	0.5%	1%
<i>Sinapis alba</i> , root length ^a	▲	▲	●	▲	▲	▲
<i>Daphnia magna</i> , toxicity ^a	●	▼	●	▼	●	●
Basal respiration ^a	●	▼	▼	▼	●	●
Microbial biomass carbon, C _{mic} ^a	▼	▼	▼	▼	▲	▲
Microbial quotient, qCO ₂ ^a	▲	●	●	▼	▼	▼
Dehydrogenase activity	▲	▲	●	●	▲	▲
β-glucosidase activity	▲	●	●	▼	▲	▲
Acid phosphatase activity	▼	▼	▼	▼	▲	▲
Urease activity	●	▲	▼	▼	▲	▲
FDA activity	▲	▲	●	▲	▲	▲
<i>Eisenia foetida</i> , body weight	●	n.d.	▲	▲	●	▲
<i>Eisenia foetida</i> , No living earthworms	●	▼	●	●	●	●
<i>Eisenia foetida</i> , No cocoons	▲	n.d.	▲	▲	▲	▲

Symbols ▲ and ▼ stand for a corresponding decrease or increase of the parameter relatively to NA soil. Symbol ● is corresponding to zero-effect. Underlined symbols () represent changes in parameters that are considered deleterious. n.d. – not detected.

^a For the data see Pukalchik et al. (2017).

idea that integral approach for soil assessment coupled with chemical measuring, biological and biological tests is crucial for monitoring the contaminated sited, and choosing of the best management practice option for soil remediation (Lors et al., 2010; Gonzalez et al., 2011).

5. Conclusions

The results of amendments' application to affect TE's mobility, along the changes in soil enzymes activities and ecotoxicity to earthworms, demonstrate that adding BC, WA or HS at relatively low doses can restore the quality of multi-contaminated soils. Moreover, high earthworm mortality occurred in BC 5% treatments, and significant depression in enzyme activity was detected in amended WA 6% soil compared to the non-amended (contaminated) samples.

This case study provides evidence, that an incorrect choice of amendment dose can exacerbate soil toxicity despite of immobilizing the trace metals. However, our results should be very carefully generalized as all the amendment materials tested, particularly BC and HS, may drastically vary in their structure and composition between the particular sources and therefore, their impact on soil properties may vary as well. The major advantage of the proposed approach is in drawing a multidimensional picture with most important variables taken into account. Thus, it should be neither overlooked nor dismissed as it brings the added value for sustainability and ecologically friendly remediation management.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at

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