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Original Research

Effect of Plant Growth on Total Concentrations of Zn, Pb, and Cd, and Their Distribution between Operational Fractions in the Upper Layer of a 100-Year-Old Zinc-Lead Waste Heap

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Abstract

This study determined total concentrations and operational fractions of Zn, Pb, and Cd from sequential extraction in samples originating from the 0-15 cm upper layer of a 100-year-old calamine waste heap in Bolesław, Poland. Also investigated was the accumulation of the heavy metals in the tissues of *Biscutella laevigata* plants growing on the heap, and microbial activity (number and enzymatic activities) in the samples. Sequential extractions of heap material indicated that the exchangeable fraction (considered as bioavailable) of all the tested heavy metals was less than 0.5% of their total concentrations. Plant growth was found to have an effect on soil organic matter accumulation, number of fungi, enzymatic activity, and distribution of Zn, Pb, and Cd between operational fractions in the heap material. The number of isolated microorganisms and their enzymatic activities in samples with plant cover were almost the same as or higher than those in non-contaminated soils under vegetation with similar physico-chemical properties, as studied by other authors.

Keywords: soil organic matter accumulation, Zn, Pb, Cd plant accumulation, microbial and enzymatic activity

Introduction

There is a general interest in long-term effects of plant growth on the toxicity and mobility of heavy metals (HM) introduced at high concentrations into the environment [1]. In Poland, long-term HM-polluted sites are found in the southwestern Kraków-Częstochowa Upland near Bolesław and Olkusz. They are 100-year-old calamine waste heaps in Bukowno, a large mining and steel mill, and Bolesław, a steel mill, containing waste from extraction and smelting of Zn-Pb ores, casted without distinct horizons [2, 3].

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Heavy metals cannot be degraded and their mobility, bioavailability, and potential toxicity in the soil depend on their concentration in soil solution. The latter is the net result of the sorption/desorption processes occurring among the different components of the soil solid phase, such as clay, organic matter, and iron-, manganese-, and aluminum oxides, under the influence of biotic and abiotic factors [4]. It is well known that microbial activity is an essential factor affecting the properties of the aqueous phase of soil. The largest numbers of microbes and their highest physiological activity are found in rhizosphere soil enriched with root exudates [5]. Heavy metals affect the growth, morphology, and metabolism of microorganisms in soil through functional disturbance, protein denaturation, or destruction of the integrity of cell mem-

branes [6]. Soil microbial activity has been proposed as a useful indicator of soil improvement or soil degradation. Soil enzyme activities are considered as sensitive early indicators of both natural and anthropogenic disturbance [7, 8]. On the other hand, exposure to HM may lead to the selection of a metal-tolerant population of microorganisms [9].

Plants accumulate metals in their tissues, and their growth can stabilize metals in soils [10] as well as enhance retention of elements by binding them with phenolics [11]. On the other hand, plant growth can enhance metal mobility by decreasing pH and metal chelation by root exudates [12]. Vegetation can protect the soil surface from dispersal of HM by wind and water erosion. Elevated evaporation reduces the flow of water through the soil, thus decreasing the amount of metals released from solid phase that reaches the ground and surface waters [13]. The surface of the 100-year-old waste heap in Bolesław was found to have two types of sites, those covered with spontaneously developed plant growth (SHR) as well as those with a lack of plant growth (SH). No data are available on how long and why some of the sites had no plant growth.

The aim of the present study was to examine the effect of plant growth on:

1) the physico-chemical characteristics of the heap material
2) the total concentrations of Zn, Pb, and Cd and their concentrations in the operational fractions from sequential extraction
3) microbial numbers, by comparing the values of these parameters in heap material taken from the SHR site (with plant cover) and the SH site (without plants)

Also, concentrations of these heavy metals in the tissues of the metallophyte *Biscutella laevigata* growing on the heap and activities of enzymes (dehydrogenase, cellulase, and xylanase) as indicators of HM toxicity were measured.

Materials and Methods

Characteristics of Study Area and Heap Material

The study area (a mine spoil) is located in the industrial region of southern Poland (Bolesław, near Olkusz, Upper Silesia). In this area, deposits of zinc and lead ores are found in Triassic beds, chiefly in ore-bearing dolomites. These ores mainly contain zinc and lead sulfides, zinc and lead carbonates, silicate and oxidized iron minerals, and significant amounts of Cd, Ge, Tl, and Ag. Silver and lead have been mined and processed in this region since the 13th century, while zinc has been smelted since the 18th century [2, 3].

The age of the studied waste heap is estimated at 100 years. This area is an artificial, irregular heap, strongly stony with a non-differentiated profile of initial soil. The surface of the heap is covered with a few-centimeter-thick humus layer with dense vegetation cover, but many places have no humus layer or plant vegetation. The plant cover consists of specific calamine plants such as *Armeria hallerii*, *Biscutella laevigata*, *Silene vulgaris*, *Reseda lutea*, *Gypsophila fastigata*, *Erysinum pannonicum*, *Cerastium arvense*, and others [3].

Table 1. Characteristics of the heap material from SHR and SH sites.

Characteristic	SHR	SH
pH (H_2O) [1]	7.2±0.03	7.3±0.15
Total organic C ($g \cdot kg^{-1}$) [1]	48.3±3.9	15.0±3.0
% of skeleton particles (>1.0 mm) [2]	46±2.3	64±5.5
Texture of particles <1.0 mm (=100%) [2]		
Sand (1.0-0.1 mm)	42±2.5	44±4.0
Silt (0.1-0.02 mm)	29±1.5	23±1.8
Clay (<0.02 mm)	29±1.7	33±1.3
Total concentration of metals ($mg \cdot kg^{-1}$) [2]		
Zn	51,687±1 632	43,690±5 235
Pb	5,382±1 145	3,225±74
Cd	342±8	282±31

[1] Results are mean values of 6 replicates;
[2] Results are mean values of 3 replicates.
Standard deviations are shown as ± S.D.

The samples of heap material were taken at selected sites on one of the 100-year-old waste heaps originating from a non-ferrous smelter. Part of the sites had dense plant cover (SHR), and the remaining ones had no vegetation (SH). The material was sampled from 0 to 15 cm of the upper layer of the heap in the third week of October 2007 and was passed through a 1-mm sieve to separate skeleton particles (stones and gravel) from the fine fractions (sand, silt, and clay). The physico-chemical characteristics presented in Table 1 are representative of <1-mm fractions. Texture of this material was determined using the aerometric method of Casagrande modified by Prószyński and supplemented with the sieve method to determine the sand fraction [14]. Values of pH were determined in a soil suspension in water (1:2.5), after 1 hour of standing and brief stirring using a Beckman pH-meter [15]. Organic carbon content was analyzed according to Tiurin's method [14].

Sequential Extraction of Zn, Pb, and Cd from Heap Material and Accumulation in Plants

Deionized water was used throughout this study. All glassware and plastic containers were soaked in 7.5 M HNO_3 for at least 2 hours and rinsed thoroughly with deionized water before use.

Heap material was sequentially extracted according to Keller and Vedy [16], modified by Majewska et al. [17]. To determine the operational fractions of Zn, Pb, and Cd, 1 g samples of mixed heap material taken from 9 locations were extracted with 0.1 M $NaNO_3$ (10 ml), 1 M $NH_3OH \cdot HCl$ in 25% CH_3COOH (20 ml), a mixture of 30% H_2O_2 + 0.02 M HNO_3 (5 ml + 3 ml), and aqua regia – a mixture of con-

centrated HCl (3 ml) + HNO_3 (2 ml). This procedure was repeated three times for each mixed sample. Concentrations of Zn, Pb, and Cd were measured in each fraction with an atomic absorption spectrophotometer (Unicam 939AA Spectrometer).

Biscutella laevigata was collected on the third week of October 2008. Roots and shoots of the plants were rinsed three times with deionized water to remove heap material. Plant dry matter was determined after drying at 105ºC until constant weight. In order to determine metal concentrations in plant tissues, samples of dry plant material (20 mg) were digested with concentrated HNO_3 for 12 hours at room temperature. Next, HNO_3 was evaporated to dryness, and then the residue was dissolved in 1 M HNO_3. Concentrations of Zn, Pb, and Cd were measured with an atomic absorption spectrophotometer (Unicam 939AA Spectrometer).

Determination of the Number of Microorganisms

The number of microorganisms was estimated by the plate count method. Copiotrophs were cultivated on PYS agar, oligotrophs on this medium diluted 100-fold, *Pseudomonas* species on King's B agar [15], cellulose-decomposing microorganism on agar with Avicel (Sigma-Aldrich) as a C-source [18], pectin-decomposing microorganisms on agar with pectin (Sigma-Aldrich) as a C-source [19], and fungi on Martin medium [20]. The cultivations were performed for 5-14 days at 28ºC. The numbers of microorganisms were expressed as colony forming units (CFU) per gram of dry weight of heap material.

Enzyme Activity

Dehydrogenase activity in the heap material was measured by the 2,3,4-triphenyl tetrazolium chloride (TTC) reduction method according to the procedure described by Casida [21] and expressed as μg of formazan produced per gram dry weight of heap material per 24 hours. Carboxymethyl cellulose sodium salt (CMC, Sigma-Aldrich) was used as the substrate for endoglucanase, Avicel (Sigma-Aldrich) for total cellulases (endo- and exoglucanase and β-glucosidase activity), and xylan (Sigma-Aldrich) for xylanase. The amounts of reducing sugars released from cellulose or xylan (glucose or xylose, respectively) after its incubation with SHR or SH materials were measured according to the method of Nelson and Somogyi [15]. The concentrations of the measured compounds were determined with a UV-visible spectrophotometer (Varian) and expressed as μg per gram dry weight of heap material during 1 hour.

Measurement of Organic Compound Concentrations

The amounts of organic compounds (total Fe(III)-chelators, siderophores, phenols, citric acid, and proteins) were measured in deionized water and 0.1 M $NaNO_3$ extracts of heap material. The total amount of Fe(III)-chelators was measured in reaction with $FeCl_3$ using desferrioxamine B as the standard [22]. Hydroxamate siderophores were determined by the Csaky method with $NH_2OH{\cdot}HCl$ as the standard [23]. The concentration of catechol siderophores was determined by the Arnow method with 3,4-dihydrohybenzoic acid as the standard [24]. Total proteins were measured by reaction with the Bradford reagent with albumin as the standard [25]. The Folin-Ciocalteau reagent was used to determine total soluble phenolic compounds with ferulic acid as the standard [26]. The concentration of citric acid was measured by the UV-method for the determination of citric acid in foodstuffs and other materials (R-BIOPHARM Enzymatic Bioanalysis, Cat. No. 10 139 076 035). The concentrations of organic compounds were measured with a UV-visible spectrophotometer (Varian).

Data Analysis

All chemical, microbial, and enzymatic analyses were performed on samples of mixed heap material taken from 9 locations. The physico-chemical characteristics of the heap material, total concentrations of Zn, Pb, and Cd in the heap material and plant tissues, and their concentrations in individual operational fractions are given as mean values of three replicates. Values of pH, organic carbon content, and concentrations of organic compounds are means of six replicates and enzymatic activity is a mean of 12 replicates. Data in the table are presented as means plus/minus standard deviation (± S.D.). In Fig. 1, standard deviations are shown as deviation bars.

Results

Total Zn, Pb, Cd Concentrations, Soluble and Exchangeable Fractions, and Accumulation in Plant Tissues

The calamine waste heap material sampled in 2007 from two types of sites, SH without plant cover and SHR overgrown with dense plant cover as a result of spontaneous succession, was alkaline (pH 7.3 and 7.2, respectively). The average concentrations of tested HM in the material from both types of sites were high (Table 1). The total concentrations of Cd, Zn, and Pb in SHR samples were 18%, 15%, and 40% higher, respectively, than those measured in SH samples. There was no significant difference in the amounts of exchangeable Zn, Pb, and Cd extracted with 0.1 M $NaNO_3$ from SHR and SH samples. This fraction of the tested HM did not exceed 0.5% of their total concentrations in either material (Table 2).

The amounts of Zn, Pb, and Cd in the soluble and exchangeable fractions in samples from the Bolesław heap were also compared to the amounts of these elements accumulated by *Biscutella laevigata*. The plants harvested from the SHR site accumulated larger amounts of Zn and Pb in their aboveground parts (4,581 $mg{\cdot}kg^{-1}$ and 447 $mg{\cdot}kg^{-1}$,

respectively) than in their roots (2,930 mg·kg^{-1} and 331 mg·kg^{-1}, respectively), but the same amounts of Cd in both plant parts (58 mg·kg^{-1}). Plants originating from the waste heap in Bolesław accumulated in their roots no more than 0.3% dry mass of Zn, 0.033% of Pb, and 0.006% of Cd.

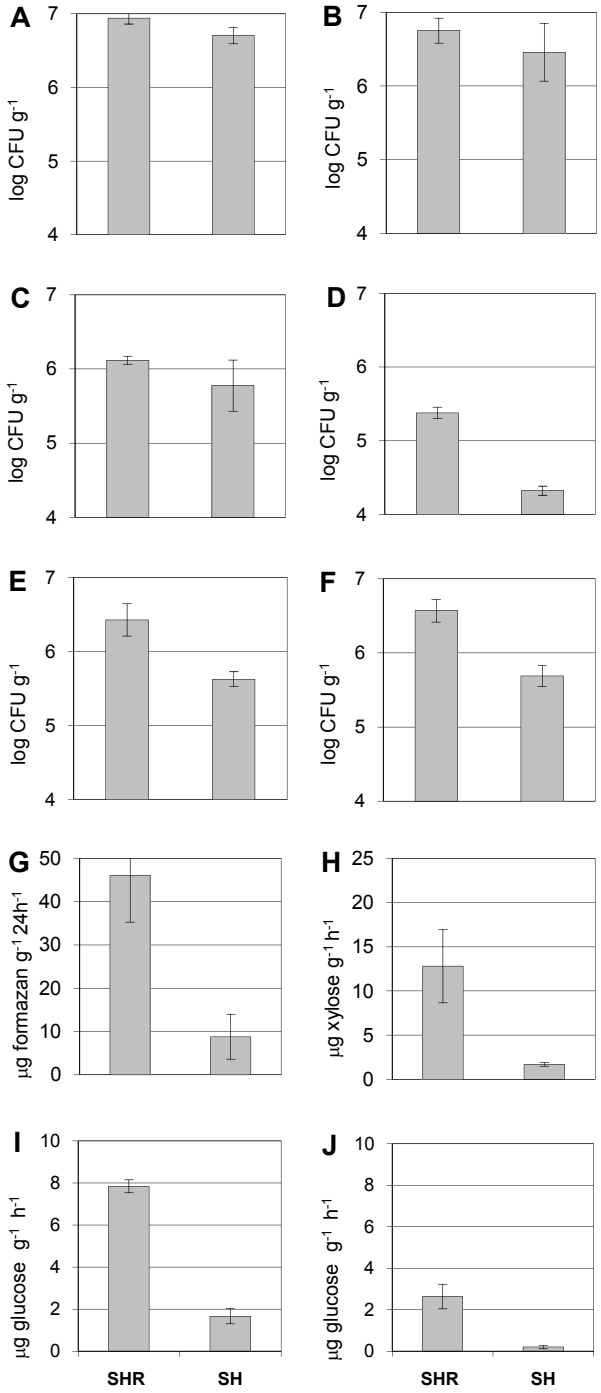

Fig. 1. Numbers of microorganisms isolated from heap material from SHR and SH sites and their enzymatic activities. Results are mean values of 12 replicates. Standard deviations are shown as deviation bars.
A – copiotrophs, B – oligotrophs, C – *Pseudomonas* sp., D – fungi, E – cellulolytic microorganisms, F – pectinolytic microorganisms, G – dehydrogenases, H – xylanases; I – total cellulases, J – endoglucanases

Microbial and Enzymatic Activity

The numbers of bacteria and fungi in SHR and SH samples were high (Fig. 1) and, as generally observed, their growth was significantly stimulated by plant cover (microbial counts increased on average 4-fold for bacteria and 11-fold for fungi). Also, the well-known enhancing effect of plant cover on soil enzymatic activity was noted. The activities of dehydrogenases, xylanases, total cellulases, and endoglucanases were on average about 5, 8, 5, and 13 times higher, respectvely, in SHR than in SH samples (Fig. 1).

Extraction of Zn, Pb, Cd and Organic Compounds

Sequential extraction of the heap material indicated that the distribution among operational fractions was significantly different for the individual HM (Table 2). However, the soluble and exchangeable fractions of all the tested HM (Zn, Pb, and Cd) constituted less than 0.5% of the total concentrations of these elements in the heap material. The largest amounts of Zn were found in the residual fraction, while Pb and Cd were most abundant when bound to Fe and Mn oxides. There was a significantly higher percentage of Zn and Cd bound to organics in SHR than in SH samples. It has to be noted that although the percentages of the individual fractions in SHR and SH samples were similar, the concentrations of the individual elements in the fractions extracted from SHR were significantly higher than those extracted from SH. Chemical analyses of water and 0.1 M $NaNO_3$ extracts of SHR and SH samples revealed that a water extract of SH samples was significantly enriched with proteins. Extraction of the heap material with 0.1 M $NaNO_3$ showed the presence of citric acid and a 3-fold higher concentration of total chelators in SHR than in SH samples (Table 3).

Discussion

B. laevigata is a perennial plant native to mountainous regions of Europe, which prefers habitats with levels of HM higher than natural. In Poland, this plant grows in the western Tatra Mountains, and in lowlands it is only found on calamine waste heaps in the vicinity of Olkusz and Bolesław. It is a pioneer plant on mine spoils [2]. Studies conducted in the Austrian Alps [27] and in southern France [28] showed that *B. laevigata* shoots contain up to 1,090 mg·kg^{-1} of lead, up to 78 mg·kg^{-1} of cadmium, and more than 1.5% of dry matter of thallium. The results of those studies allowed researchers to classify this species as a specific hyperaccumulator of Pb and Tl. According to the generally accepted definition by Baker and Brooks [29], plants accumulating more than 1,000 mg·kg^{-1} dry mass of Pb and over 1% of numerous other metals in their tissues can be considered hyperaccumulators. However, the ability of *B. laevigata* to hyperaccumulate HM, as found in the plants grown in France [28] and the Austrian Alps [27], was not confirmed by authors analyzing this plant species growing

Table 2. Operational fractions from sequential extraction of heavy metals in the heap material from SHR and SH sites.

Sites	Metals fractions	Zn		Pb		Cd	
		mg·kg^{-1} dry weight (% of total concentrations)					
SHR	Soluble and exchangeable	85±53	(0.16%)	25±4	(0.47%)	1.2±0.2	(0.36%)
	Bound to Fe and Mn oxides	6,129 ±31	(11.86%)	3,590±659	(66.92%)	214.8±8.1	(62.78%)
	Bound to organics	178±30	(0.34%)	4±2	(0.07%)	6.0±0.8	(1.74%)
	Residual	45,296±1,580	(87.63%)	1,763±481	(32.55%)	120.1±0.4	(35.11%)
SH	Soluble and exchangeable	55 ±39	(0.13%)	12±8	(0.38%)	1.1±0.2	(0.38%)
	Bound to Fe and Mn oxides	5,989±143	(13.79%)	2,174±126	(67.36%)	188.7±17.5	(67.06%)
	Bound to organics	80±29	(0.19%)	10±3	(0.31%)	2.5±0.3	(0.90%)
	Residual	37,565±5,160	(85.89)	1,030±47	(31.95%)	89.4±13.8	(31.66%)

Results are mean values of 3 replicates. Standard deviations are shown as ± S.D

on the waste heap in Bolesław. *B. laevigata* harvested from that heap in 2008 accumulated in its roots 0.3% dry mass of Zn, 0.033% of Pb, and 0.006% of Cd. Those amounts are not sufficient to classify the plants of *B. laevigata* growing on the heap in Bolesław as hyperaccumulators. On the other hand, as stated by Wierzbicka and Pielichowska [30], the smaller amounts of metals accumulated by *B. laevigata* grown on the calamine waste heaps in Bolesław may be a result not as much of the characteristics of the plant but of smaller amounts and availability of metals in soil. The pool of Zn, Pb, and Cd extracted with 0.1 M $NaNO_3$ as operational fractions of sequential extraction (considered as bioavailable) accounted, in the present study, for no more than 0.5% of their total concentrations in the heap material.

The total Zn, Pb, and Cd concentrations in the samples from the 0-10 cm upper layer of the calamine waste heaps have been measured by other authors since 1981. The plant cover in the study area had been developing with time. In 1981 the dump was stony with a non-differentiated profile resembling initial soil poorly covered with vegetation [2]. Measures done in 2002 [31] indicated that total concentrations of Zn, Pb, and Cd had increased significantly compared to those measured earlier by Godzik [2]. An analysis done in 2007 by the present authors showed a further increase in total concentrations of these elements in the 0-15 cm layer, particularly for the SHR site, where it was accompanied by an increase in organic matter contents. An increase in total HM concentrations in the upper layer of soil contaminated with Cd, Zn, Pb, Cu, and Cr after 33 years of plant growth was reported by Mertens et al. [12]. In that study, the increase, which was accompanied by enhanced organic carbon content in the upper layer of the heap, was due to deposition and decomposition of contaminated plant material. As indicated in a study by Boucher et al. [32], the decomposition of HM-contaminated plant residues follows a course with two main stages. The first one is fast and takes place after an abiotic transfer of HM (Zn and Cd) from readily soluble plant tissues onto fine soil constituents, keeping metals away from the liquid phase. At this stage, the microbial biomass and the metal content of the solid soil fractions (particularly those rich in particulate organic matter) increase. During the second stage, the metal content in the solution increases with mineralization of organic matter; however, the remaining metal-rich plant material seems to create a stable organic carbon component in the soil.

Table 3. Concentrations of organic compounds in the heap material from SHR and SH sites (mg·kg^{-1} dry weight of heap material).

Organic compounds	H_2O extract		0.1 M $NaNO_3$ extract	
	SHR	SH	SHR	SH
Total chelators	23.34±4.65	25.46±4.67	66.20±19.40	25.27±4.65
Hydroxamate siderophores	0	0	60.52±13.73	56.15±10.34
Katechol siderophores	2.05±0.01	4.11±2.06	7.77±1.43	5.01±1.04
Phenols	1.83±0.17	1.74±0.58	2.91±1.54	4.05±1.74
Proteins	8.62±3.83	18.59±2.00	16.67±2.00	16.61±7.19
Citric acid	0.0007±0.0004	0	0.007±0.0006	0

Results are mean values of 6 replicates. Standard deviations are shown as ± S.D

The relatively high number of microorganisms in the SHR material, close to that found in an unpolluted rendzina [33] also characterized by alkaline pH and a non-developed soil profile with a layer of organic matter on the surface containing from 1% to 2% of organic carbon, can be connected with the very low concentration of the exchangeable fraction of tested HM in this sample as well as selection, with time, of resistant organisms. There are controversial data concerning the tolerance of soil bacterial and fungal communities toward HM. Some authors [34, 35] have demonstrated that long-term experimental contamination of soil with HM results in higher tolerance toward these elements in fungi than in bacteria. Others [36, 37] have found that bacteria and fungi react to soil contamination with HM in the same way, but a greater sensitivity of fungal biomass relative to bacterial biomass also has been reported [38]. These various findings are probably due to differences in the chemical forms of HM, the mode of their application, the properties of soil, and the methods of detection of microbial communities used.

It is worth noting that the activities of the tested enzymes (dehydrogenases and enzymes involved in C-cycl) in SHR samples were almost the same as or even higher than those in uncontaminated Eutric Cambisol under vegetation with similar physical and chemical characteristics [39]. Soil hydrolytic activities are key factors controlling nutrient availability in soil [40]. There are reports indicating that enzymes involved in C-cycling are less affected by HM in contaminated soils than enzymes related to N, P, and S cycling [39].

The percentage of soluble and exchangeable fractions and the toxicity of Zn, Pb, and Cd in the material from the upper layer of the heap were low, and forms of these elements present in or released to solution (extracted with 0.1 M $NaNO_3$) in SHR samples were less toxic to microorganisms than those originating from SH samples. Plant growth affected the distribution of all the tested HM between operational fractions of sequential extraction. However, differences were found in the concentration patterns of the individual metals in the particular fractions. While for Pb an increase was found in its soluble, exchangeable, and Fe/Mn-oxide-bound fractions, Zn and Cd showed increased concentrations in the organically bound fraction. Important from the ecological point of view was the increase in the residual fractions of all the tested HM in samples of material from the upper layer of SHR. The increase in the soluble and exchangeable mobile forms of Pb in the SHR material suggests a possible transfer of this element to the deeper layers of the heaps. We suspect that this may be due to root exudates and microbial metabolites present in the heap material and their interaction with HM, which probably results in the formation of complexes. Free forms of metals in a solution generally are supposed to be more toxic toward microorganisms than complexed or sorbed species [41, 42]. The total concentration of chelators and citric acid were found to be higher in the material originating from SHR than in that from SH.

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