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Levels of Organic Compounds, Number of Microorganisms and Cadmium Accumulation in *Festuca ovina* Hydroponic Culture

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A b s t r a c t

Understanding the microbiological, biochemical and physiological aspects of phytoremediation of soil and water environments polluted to different degrees with heavy metals has very important theoretical and practical implications. In this study, a comparison was made between total cadmium concentration in root and shoot tissues as well as concentrations of particular fractions of Cd immobilized by roots of *Festuca ovina* (Sheep's fescue) hydroponically cultivated in nutrient solutions supplemented with 1 µg Cd ml^{-1} and those cultivated at 10 µg Cd ml^{-1}. After three weeks of *F. ovina* cultivation, the number of bacterial CFU and the amounts of organic chelators, siderophores, proteins and reducing sugars in the growth medium and on the root surface were higher at 10 than at 1 µg Cd ml^{-1}. The grass also reacted to the high Cd concentration by a decrease in plant growth and dehydrogenase activity in root tissues. The concentration of Cd determined in fractions bound with different strength in roots was significantly dependent on Cd concentration in the growth medium. When the plants were grown at 1 µg Cd ml^{-1}, 9% of the immobilized cadmium was loosely bound to the root surface, 20% was exchangeable adsorbed, and 28% was bound by chelation; at 10 µg Cd ml^{-1}, the respective values were 12%, 25%, and 20%. About 43% of the immobilized cadmium remained in roots after sequential extraction, and bioaccumulation factors in shoots had the same values independently of Cd concentration. At both Cd concentrations, the cadmium translocation index for *F. ovina* was low (< 1), which is why this grass can be recommended for phytostabilization of the metal under study.

K e y w o r d s: hydroponic culture under different Cd concentrations, metal chelators, phytoremediation, sequential extraction of Cd

Introduction

Cadmium is not essential for plant, animal and human metabolism (Van der Perk, 2006). The only known biological function of Cd has been described in marine diatoms under zinc limitation. In those conditions, a low concentration of Cd acted as a cofactor in Cd-carbonic anhydrase (Xu and Morel, 2013). Cadmium tends to be less strongly adsorbed than other divalent metals and, therefore, it is more labile in soil and more available to plants (An *et al.*, 2011; Kacálková *et al.*, 2014). Areas located in industrial regions can contain high concentrations of heavy metals (HM). For example, in southern Poland, there are regions with HM-polluted sites (*e.g.* Bolesław near Olkusz, Upper Silesia) where the concentrations of Zn, Pb and Cd exceed 40,000 mg kg^{-1}, 5,000 mg kg^{-1} and 300 mg kg^{-1}, respectively (Majewska *et al.*, 2011). In such cases, it is very important to prevent the migration of these metals to surrounding uncontaminated soils. Apart from physico-chemical stabilization (compost applica-

tion, liming *etc.*), phytostabilization can be taken into account as a method of remediation of HM-contaminated soils (Alvarenga *et al.*, 2008). This method is an aesthetically pleasing strategy that uses the ability of metal-tolerant plants to grow in and cover contaminated ground. Phytostabilization consists in sequestration of metals within roots and the rhizosphere without translocating them into the above-ground plant tissues (Mendez and Maier, 2008; Ganesan, 2012; Zhang *et al.*, 2012). Plants are colonized by endophytic and rhizospheric microorganisms which increase the resistance of their hosts to environmental stresses (Jha *et al.*, 2013). These microorganisms can play an important role in phytoremediation of soils polluted with heavy metals (Soleimani *et al.*, 2010) and many other, especially organic, contaminants (Gerhardt *et al.*, 2009).

Organic components of root exudates are a good source of nutrients and energy for microorganisms, hence, a microbial community living in the rhizosphere can be 10–100 times larger than the same type of community found in bulk soil (Huang and Germina, 2002).

Microbial immobilization of a HM from rhizosphere soil solution can involve binding of the metal by cell envelopes, its intracellular accumulation, formation of insoluble complexes with extracellular biopolymers or precipitation with inorganic anions, such as sulfides and phosphates (Kurek and Majewska, 2012). On the other hand, microorganisms can accelerate the decomposition of organic matter (*e.g.* dead roots) and, consequently, mobilize HM in the rhizosphere. Dissolved organic ligands (*e.g.* carboxylates and phenolics) exuded by plant roots and those produced by microorganisms are also responsible for HM mobilization. Soluble ligands which have a higher affinity for HM than roots and other solids, and those ligands which acidify the root zone are important factors in increasing HM desorption (Haoliang *et al.*, 2007; Kim *et al.*, 2010; Kurek and Majewska, 2012). These processes can increase HM concentrations in soil solution and decrease the capacity of phytostabilization.

The aim of the present study was to compare speciation of Cd (water-soluble, exchangeable absorbed and chelate-bound fractions) immobilized by roots of *Festuca ovina* grown at 1 or 10 µg Cd ml^{-1} of the growth medium. Also, the numbers of microorganisms inhabiting the growth medium and the root surface, and the concentrations of organic compounds (*i.e.* phenols, proteins, citric acid, reducing sugars, total Fe(III)-chelators and siderophores) released by roots and/or root-associated microorganisms after three weeks of plant cultivation in both media were compared.

Experimental

Materials and Methods

Plant growth conditions. Half a gram of non-sterilized grass seeds (about 625) were transferred to sterile plastic sieves with a surface area of 170 cm^2. The sieves were placed in sterile plastic containers (2,000 ml) holding 600 ml of autoclave-sterilized (non-aerated) Hoagland medium (Atlas, 1995; Sipos *et al.*, 2013) supplemented with 1 or 10 µg Cd ml^{-1} (as CdCl$_2$·2.5H$_2$O). Cadmium-free Hoagland medium was used as a control. All glassware and plastic containers were soaked in 7.5M HNO$_3$ for at least 2 h, rinsed thoroughly with deionized water and sterilized before use.

The plants were grown in a growth chamber (Biogenet, FD147 Inox) at 24/18°C (day/night), with a 12/12 h (day/night) photoperiod, at a photosynthetic photon flux density of 250 µmol m^{-2} s^{-1} for 3 weeks. The volume of the growth medium was kept constant by replenishing the volume evaporated during plant growth with sterile Hoagland medium without added Cd (Majewska and Kurek, 2011). After 3 weeks, 30 randomly-selected plants were removed from the medium and separated into roots and shoots. The roots were washed with sterile deionized water. Shoot height and root length as well as fresh and dry weight of the plants were determined. The dry weight of the plants was estimated after heating the plant material at 105°C until a constant weight was obtained.

Estimation of cadmium concentration in growth medium and in plants. The growth medium was collected and centrifuged (9,000 × g for 15 minutes), and its pH was measured using a Beckman pH-meter. The concentration of cadmium ions was determined using a cadmium ion selective electrode from SENTEK with a detection limit of 10^{-7} M. Next, 20 ml of the medium was evaporated to dryness and digested with concentrated HNO$_3$. In order to determine the concentration of the metal in shoots and roots, 30 mg of dry plant material was digested with concentrated HNO$_3$. Next, the acid was evaporated to dryness, and then the residue was dissolved in 10 ml of 1M HNO$_3$ (Majewska *et al.*, 2006). Cadmium concentrations were determined by mixing 1 ml of mineralizate, 2 ml of 3 M NaOH, 2 ml of an aqueous solution of Triton X-100 (5%) containing 0.3 mM dithizone, and 5 ml deionized water. The absorbance of the samples was determined at λ = 549 nm using a Varian UV-visible spectrophotometer (Fiedler *et al.*, 2004; Majewska and Kurek, 2011). The bioaccumulation factor (BAF) was calculated as Cd concentration in roots or shoots (mg kg^{-1}) divided by the initial concentration of Cd in the medium (mg l^{-1}), and the translocation index (TRL) was described as mg Cd kg^{-1} of dry weight of shoots divided by mg Cd kg^{-1} of dry weight of roots (Mendez and Maier, 2008; Cheraghi *et al.*, 2011).

Determination of dehydrogenase activity in root tissues. Metabolic activity in root tissues was assessed by the tetrazolium chloride (TTC) reduction method according to the procedure described by Comas *et al.* (2000). In the tissues, TTC was reduced to formazan (a bright-red pigment) by dehydrogenase enzymes. Roots (50 mg of fresh weight) were submerged in 3 ml of 0.6% TTC in 0.05 M Na$_2$HPO$_4$–KH$_2$PO$_4$ buffer (pH 7.4) containing 0.05% Triton X-100. The samples were incubated at 28°C for 24 h, rinsed twice with distilled water, and extracted with 10 ml of 95% ethanol for 10 minutes in a water bath at 85°C. The total volume of the extract was made up to 25 ml, and the absorbance was measured at λ = 490 nm (Varian, UV-visible spectrophotometer). Control roots were boiled for 15 minutes in distilled water to denature the enzyme. Dehydrogenase activity in roots was expressed as µg of formazan formed by 1 mg of root dry weight.

Sequential extraction of Cd immobilized in roots. After three weeks of plant growth, speciation of the metal immobilized by roots was analyzed using a three-

step sequential extraction procedure. This method makes it possible to fractionate metals into fractions operationally defined as the water-soluble fraction (extracted with deionized water), the exchangeable absorbed fraction (extracted with $0.1\,M\,NaNO_3$) and the chelate-bound fraction (extracted with $0.02\,M$ EDTA). In addition, a residual fraction of cadmium, not removed during sequential extraction, was determined (Majewska and Kurek, 2011). Roots (500 mg fresh weight) were suspended in 100 ml of each extractant and shaken (250 rotations per minute) at room temperature for 30 minutes. The extracts were evaporated to dryness, and Cd concentration was determined in the residues as described above. After sequential extraction, the roots were collected by filtration, dried and digested with HNO_3, and the amount of Cd in root tissues was determined. Total cadmium concentrations as well as Cd ions were determined in all of the tested fractions as described above.

Determination of microorganisms CFU number. After three weeks of plant growth, the numbers of microorganisms in the growth medium, those released from the root surface during sequential extraction and those inhabiting interior root tissues were estimated by the plate count method. Fungi were cultivated on Martin medium (Martin, 1950) and bacteria on nutrient agar (Atlas, 1995) for 7 days at 28°C. The number of microorganisms was expressed as $\log_{10}$ colony forming units (CFU) per milligram of dry weight root mass.

Determination of organic compound concentrations. Dissolved organic compounds, such as reducing sugars, proteins, phenols, citric acid, total Fe(III)-chelators and siderophores were quantitated in water extracts and $0.1\,M\,NaNO_3$ extracts from roots, and in the growth medium. Before determination of organic compounds, the plant growth medium was centrifuged ($9,000 \times g$ for 15 minutes). The amounts of reducing sugars were measured according to the method of Nelson and Somogyi (Alef and Nannipieri, 1995). Bradford reagent was used to determine protein concentrations, with albumin as a standard (Bradford, 1976). Total soluble phenolic compounds were measured by reaction with the Folin-Ciocalteau reagent, and calibration was performed using ferulic acid as a standard (de Ascencao and Dubery, 2003). Citric acid was determined using a commercial kit (R-BIOPHARM Enzymatic Bioanalysis, Cat. No 10 139 076 035). Reactions with $FeCl_3$ were carried out to estimate the total amount of organic Fe(III)-chelators. In this method, desferrioxamine B was used to construct the calibration curve (Atkin *et al.*, 1970). Catechol and hydroxamate siderophores were determined by the Arnow method with 3,4-dihydroxybenzoic acid as a standard (Arnow, 1937) and the Csaky method with hydroxylamine hydrochloride (Csaky, 1948), respectively.

Data analysis. Three experimental settings were used for each treatment (0, 1 and $10\,\mu g\,Cd\,ml^{-1}$ of growth medium), and the experiment was performed three times. One-way analysis of variance was carried out to compare the mean values. When significant p-values were obtained ($p < 0.05$), differences between the individual means were compared using Student's t-test (Brandt, 1999). All data analyses were performed using Microsoft®Excel 2010.

Results

Plant growth and dehydrogenase activity under different Cd concentrations. Symptoms of cadmium toxicity to grass seedlings were developed 3 weeks after the treatments, especially in the presence of $10\,\mu g$ $Cd\,ml^{-1}$ in the growth medium. Yellow lesions on shoots and dark-brown root tips were found. Cd concentration in the growth medium did not affect the number of plants surviving at the end of the experiment (Table I). Plant growth, expressed as shoot height and shoot fresh weight, was significantly inhibited when the plants were exposed to $1\,\mu g\,Cd\,ml^{-1}$. Cd had a stronger effect on shoots than on roots (Table I). No significant reduction in fresh and dry weights and length of roots in the presence of $1\,\mu g\,Cd\,ml^{-1}$ was observed. Inhibition of plant growth by $10\,\mu g\,Cd\,ml^{-1}$ of the medium was much more severe than that caused by $1\,\mu g\,Cd\,ml^{-1}$. The activity of dehydrogenase enzymes in root tissues declined with the increase in Cd concentration in the growth medium (Table I). The highest decrease in the activity of these enzymes was observed in roots of plants cultivated in the presence of $10\,\mu g\,Cd\,ml^{-1}$.

Cd concentrations in shoots and roots. The concentration of cadmium immobilized by the plant biomass increased with the increase in Cd concentration in the growth medium, and was always higher in roots than in shoots. Cd concentration in roots of plants grown at $1\,\mu g\,Cd\,ml^{-1}$ was approximately three times lower than in roots of plants grown in the medium containing $10\,\mu g\,Cd\,ml^{-1}$ whereas for shoots this difference was almost 10-fold (Table I). The value of the bioaccumulation factor (BAF) for roots of plants cultivated in the medium containing $10\,\mu g\,Cd\,ml^{-1}$ was significantly lower than for those grown at $1\,\mu g$ $Cd\,ml^{-1}$. On the other hand, there was no significant difference between the values of BAF for shoots from the two treatments (Table I). Cadmium concentration in the growth medium significantly affected the values of TRL. For plants cultivated in the growth medium at $10\,\mu g\,Cd\,ml^{-1}$, TRL was 3.5 times higher than for plants cultivated at $1\,\mu g\,Cd\,ml^{-1}$ (Table I).

Concentrations of Cd in the growth medium and in the individual fractions of the root extraction. At

Table I

Effect of Cd on *F. ovina* growth parameters, dehydrogenase activity and Cd accumulation in its roots and shoots. Mean values with marked the same letter within the same row do not differ significantly ($p > 0.05$). Values are expressed as mean $\pm$ S.D. ($n = 9$)

Parameters		Cadmium concentration in the growth medium ($\mu g\ ml^{-1}$)		
		0	1	10
Number of plants		509 ± 27 a	536 ± 41 a	505 ± 28 a
Shoot height (mm)		95 ± 9 a	63 ± 19 b	49 ± 14 b
Root length (mm)		91 ± 30 a	65 ± 31 a	16 ± 7 b
FW (mg) Biomass of 100 plants	Shoots	1046 ± 106 a (100%)	700 ± 76 b (67%)	297 ± 48 c (28%)
	Roots	501 ± 68 a (100%)	602 ± 81 a (120%)	53 ± 14 b (11%)
DW (mg) Biomass of 100 plants	Shoots	148 ± 16 a (100%)	128 ± 9 a (86%)	79 ± 1 c (53%)
	Roots	57 ± 11 a (100%)	58 ± 20 a (102%)	24 ± 6 b (42%)
Activity of root dehydrogenase (μg formazan mg^{-1} DW)		2.78 ± 0.74 a	1.58 ± 0.96 a	0.52 ± 0.16 b
Cd concentration (μg Cd mg^{-1} DW)	Shoots	0	0.07 ± 0.01 b	0.76 ± 0.17 a
	Roots	0	1.74 ± 0.18 b	5.39 ± 0.30 a
BAF	Shoots	–	71 ± 12 a	76 ± 17 a
	Roots	–	1742 ± 188 a	539 ± 35 b
TRL		–	0.04 ± 0.01 b	0.14 ± 0.04 a

– not determined, FW – fresh weight, DW – dry weight, BAF – bioaccumulation factor, TRL – translocation index

Table II

Distribution of Cd between plant biomass (Cd bioaccumulation), sediment (Cd precipitation) and growth medium (soluble complexes and ionic form of Cd) after 3-week cultivation of *F. ovina* in the hydroponic system. Values are expressed as mean $\pm$ S.D. ($n = 9$)

Parameters		Cadmium concentration in growth medium ($\mu g\ ml^{-1}$)	
		1	10
Initial amount of Cd in the hydroponic system (μg)		527 ± 52 (100%)	5917 ± 42 (100%)
Amount of Cd accumulated by plants		357 ± 41 (64%)	841 ± 67 (16%)
Amount of Cd in sediment (insoluble forms of Cd)		35 ± 2 (6%)	661 ± 112 (13%)
Amount of Cd in growth medium	Ionic form	17 ± 3 (3%)	3550 ± 148 (60%)
	Non-ionic form	147 ± 3 (27%)	687 ± 48 (11%)

$1\ \mu g$ Cd ml^{-1}, plants accumulated 64% of Cd present in the growth medium, 6% was converted to insoluble forms, which were separated by centrifugation, and 30% of Cd remained in the growth medium. The metal in the medium was either in the ionic form or bound to soluble compounds (Table II). When the concentration of Cd was increased to $10\ \mu g$ Cd ml^{-1}, 16% of the metal was accumulated in the plant biomass, 13% was stored as insoluble forms and 71% was still found in the medium, primarily in the ionic form (Table II).

A three-step sequential extraction of the Cd immobilized by grass roots was applied to evaluate the participation of the dissolution (fraction 1, Cd extracted with deionized water), ionic exchange (fraction 2, Cd extracted with 0.1 M $NaNO_3$) and chelation (fraction 3, Cd extracted with 0.02 M EDTA) processes, as well as intracellular accumulation (fraction 4, residual Cd) in cadmium immobilization by roots of sheep's fescue plants. When Cd concentration in the medium was $1\ \mu g\ ml^{-1}$, 9% was extracted with deionized water, 20% was extracted with 0.1 M $NaNO_3$ and 28% with 0.02 M EDTA (Fig. 1B). The increase in metal concentration in the medium from 1 to $10\ \mu g$ Cd ml^{-1} resulted in an elevated concentration of Cd in all the fractions tested (Fig. 1A). The percentage distribution of Cd between the fractions was also changed: it increased in the water-soluble (12%) and ionic exchange (25%) fractions and decreased in the fraction extracted with 0.02 M EDTA
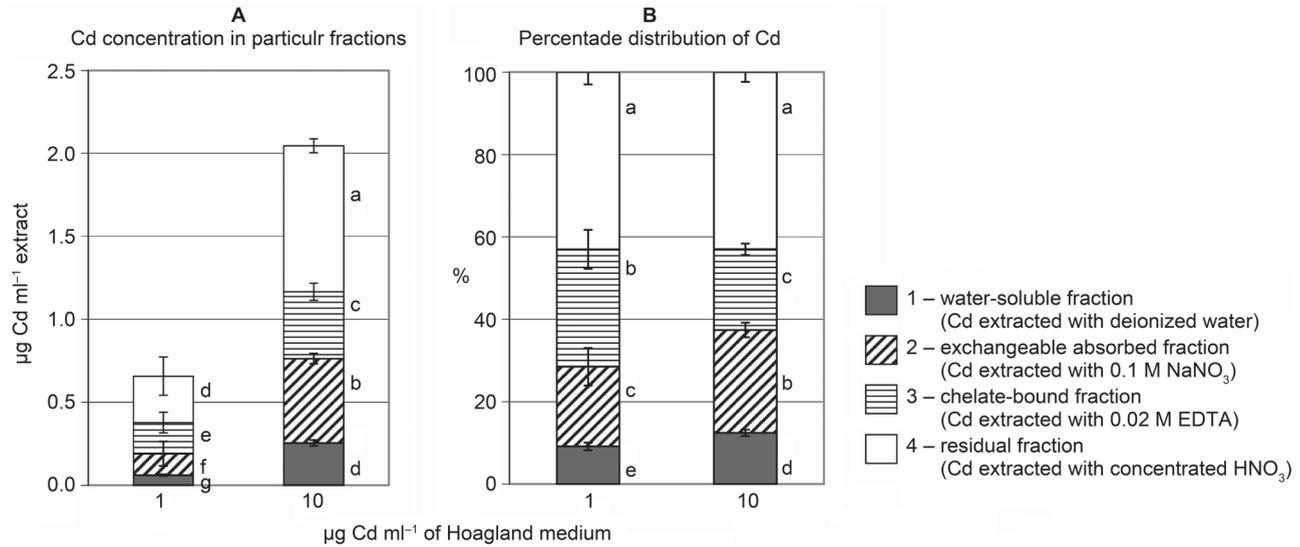
Fig. 1. Sequential extraction of Cd immobilized by roots of *F. ovina* after 3-week cultivation in Hoagland's growth medium supplemented with 1 or 10 μg Cd ml⁻¹.

A – concentration of Cd in the individual fractions (μg Cd per 1 ml of extract); B – percentage distribution of Cd between the fractions tested. Bars marked with the same letter are not significantly different ($p > 0.05$). Standard deviations are shown as deviation bars ($n = 9$)

(20%) (Fig. 1B). At both Cd concentrations in the growth medium, the roots of the grass accumulated an average of 43% of the immobilized metal in their tissues.

Cadmium extracted from roots was released as metal bound to soluble organic compounds and as free ions. When the metal was extracted with 0.02 M EDTA, the whole pool of cadmium removed was bound to EDTA in both treatments. At 1 μg Cd ml⁻¹, 12.3% of the metal extracted from roots with 0.1 M NaNO$_3$ was in the ionic form, whereas at 10 μg Cd ml⁻¹, as much as 48.5% of Cd was present in the extracts in this form. No cadmium ions were found in water extracts from roots of plants grown at 1 μg Cd ml⁻¹, but at 10 μg Cd ml⁻¹, about 7.1% Cd was in the ionic form (data not presented).

Concentrations of organic compounds. Sheep's fescue grass reacted to the presence of cadmium in the growth medium with an increased release of organic

compounds from roots into the growth medium. After plant cultivation, the growth medium supplemented with 10 μg Cd ml⁻¹, was enriched 3-fold with total chelators and citric acid, 4-fold with catechol siderophores and 5-fold with hydroxamate siderophores, proteins and reducing sugars compared to the control medium without added metal. At 1 μg Cd ml⁻¹, the growth medium was significantly enriched with catechol siderophores, proteins and reducing sugars only (Table III). Also, the amounts of total chelators, siderophores, proteins and reducing sugars extracted with deionized water and 0.1 M NaNO$_3$ from root surfaces were increased, but the amount of citric acid dropped with the increase in Cd concentration in the growth medium (Fig. 2). The solution of NaNO$_3$ mobilized more organic compounds from roots than did deionized water, except for total chelators and citric acid, which were efficiently released

Table III
Organic compounds in the growth medium after 3-week cultivation of *F. ovina* in Hoagland medium. The amounts of the individual compounds are expressed as μg mg⁻¹ dry weight of roots. Mean values marked with the same letter within the same row do not differ significantly ($p > 0.05$). Values are expressed as mean ± S.D. ($n = 9$)

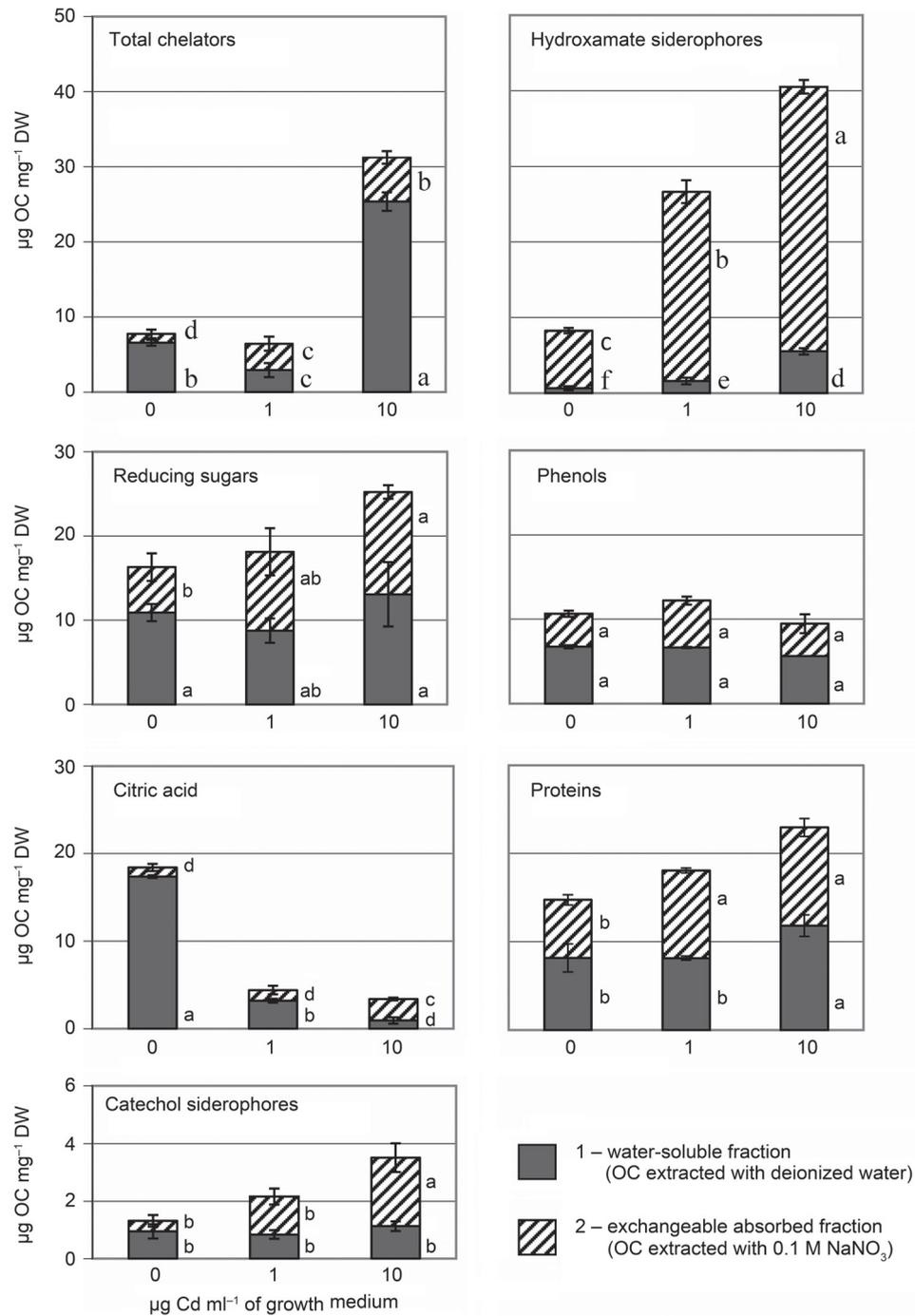| Organic compounds	Cd in the growth medium (μg Cd ml⁻¹)		
	0	1	10
Total chelators	3.43 ± 0.41 b	4.24 ± 0.31 b	11.54 ± 1.74 a
Hydroxamate siderophores	2.14 ± 1.53 b	2.65 ± 0.46 b	10.01 ± 4.06 a
Reducing sugars	2.31 ± 0.56 b	7.13 ± 1.67 a	11.84 ± 4.08 a
Phenols	0.25 ± 0.01 a	0.98 ± 0.16 a	0
Citric acids	0.90 ± 0.36 b	1.00 ± 0.18 b	2.62 ± 0.47 a
Proteins	0.72 ± 0.53 b	3.48 ± 0.62 a	3.81 ± 2.32 a
Catechol siderophores	1.50 ± 0.48 c	2.88 ± 0.18 b	5.58 ± 0.40 a

Fig. 2. Two-step sequential extraction of organic compounds (OC) from roots of *F. ovina* after 3-week cultivation in Hoagland's growth medium supplemented with 1 or 10 μg Cd ml⁻¹.

The amounts of the individual compounds are expressed as μg OC mg⁻¹ dry weight of roots. Bars marked with the same letter are not significantly different ($p > 0.05$). Standard deviations are shown as deviation bars ($n = 9$)

to the water fraction. There were no differences between the treatments in the amounts of phenols extracted from roots.

Microorganisms colonizing grass roots and the growth medium. After three-week cultivation of plants, a microbiological analysis of the plant growth media and the extracts obtained during sequential extraction of plant roots was performed. Regardless of Cd concentration in the growth medium, bacteria were the only microorganisms isolated. The number of bacteria in the growth medium without Cd added was significantly lower than in the medium supplemented with 1 or 10 μg Cd ml⁻¹ (Table IV). After sequential extraction of plant roots cultivated in Cd-contaminated media, the number of bacteria in the water and 0.1 M NaNO₃-extracts was significantly higher than in extracts from control roots (without added Cd). The concentration of Cd was without effect on the number of bacteria

Table IV

The number of bacteria in the growth medium and four sequential extraction fractions obtained from *F. ovina* roots ($\log_{10}$ CFU mg^{-1} DW of roots). Mean values marked with the same letter within a column do not differ significantly ($p > 0.05$). Values are expressed as mean ± S.D. ($n = 9$)

Cd concentration in Hoagland medium (µg Cd ml^{-1})	Bacteria in Hoagland medium	Bacteria released from roots during sequential extraction			
		Fraction 1 extracted with H_2O	Fraction 2 extracted with $NaNO_3$	Fraction 3 extracted with EDTA	Fraction 4 inhabiting root tissues
0	5.60 ± 0.12 b	5.95 ± 0.27 b	4.92 ± 0.06 b	5.13 ± 0.32 a	5.62 ± 0.05 a
1	6.02 ± 0.01 a	6.72 ± 0.21 a	5.89 ± 0.06 a	4.96 ± 0.11 a	5.23 ± 0.16 a
10	6.69 ± 0.37 a	6.96 ± 0.28 a	5.96 ± 0.03 a	5.28 ± 0.10 a	3.09 ± 0.21 b

released during extraction of roots with 0.02 M EDTA. The number of bacteria isolated from root tissues after sequential extraction was significantly decreased when Cd concentration in the plant growth medium was increased (Table IV). The water fraction was the richest in bacteria. At the concentration of 10 µg Cd ml^{-1}, the number of bacteria on the root surface (extracted with water) was 10,000 higher than the number of bacteria isolated from root tissues. Release of microbial and plant metabolites into the growth medium during cultivation of the plants resulted in a decrease in pH values from 6.6 ± 0.1 to 5.8 ± 0.3 regardless of the concentration of Cd added.

Discussion

The root surface is a site of very important processes involved in plant growth and of interactions between organic compounds and microorganisms. These interactions affect biogeochemical cycling, enhancing plant growth, and increase plant tolerance to biotic and abiotic stress (Kamaludeen and Ramasamy, 2008; Philippot *et al.*, 2013). In the present study, the grass *F. ovina* (var. Bornito – a commercially available cultivar) was used because of its resistance to drought and nutrient deficiency and because it does not require special soil conditions (as noted on packet). This choice was also influenced by observations of Grodzińska and Szarek-Łukaszewska (2009), who had found that *F. ovina* was one of the dominant plant species on postindustrial waste heaps containing zinc, lead, cadmium, thallium and other metals.

Cadmium tends to be more labile in soil and more available to plants than other divalent metals (Kacálková *et al.*, 2014). An *et al.* (2011) compared the accumulation of Cd, Pb, Cu, Cr and Fe in different plant species (tomato, maize, greengrocery, cabbage and Japanese clover herb). Among the metals tested, cadmium was easily absorbed and had the highest enrichment coefficient values for most plant tissues. In the present study, the values of the Cd bioaccumulation factor (BAF) calculated for roots of *F. ovina* growing at 1 or

10 µg Cd ml^{-1} of hydroponic solution were very high (1742 and 539, respectively) because metal retention by soil solid components was eliminated. Cadmium concentration was always higher in roots of *F. ovina* than in its shoots and increased with the elevated Cd concentration in the growth medium. A comparison of cadmium accumulation in roots and shoots of other grasses, such as *Lolium perenne* (Lou *et al.*, 2013), *Elymus elongatus* (Sipos *et al.*, 2013) and *Secale cereale* (Majewska and Kurek, 2011), also shows that roots tend to contain more of this metal than shoots. Mendez and Maier (2008), Cheraghi *et al.* (2011) and Zhang *et al.* (2012) have recommended plants (monocotyledons and dicotyledons) with a high BAF (>1) and a low translocation index (TRL <1) as the best candidates for phytostabilization. The values of TRL for *F. ovina* growing at 1 µg ml^{-1} as well as at 10 µg ml^{-1} were much lower than 1 (0.04 and 0.14, respectively). The same situation was observed in *F. ovina* growing at 5, 10, 50 and 100 µg Cd g^{-1} dry weight of soil (data unpublished).

The exposure of *F. ovina* to 10 µg Cd ml^{-1} resulted in a significant decrease in shoot height, root length, fresh and dry mass of the plants, and vitality of plant roots, while at 1 µg ml^{-1} these parameters were not significantly changed (Table I). This indicates that low concentrations of Cd may have no impact on plants. In the present study, the vitality of plant roots was determined by measuring dehydrogenase activity expressed as TTC reduction. According to Comas *et al.* (2000), there is a relationship between respiration and dehydrogenase activity, and respiration is a standard test of metabolic activity of cells. A low TTC reduction can be indicative of a generally low metabolic activity or necrosis of root tissues. TTC reduction can be evaluated in the stele but not in the cortex of old roots. Heavy metal toxicity can accelerate the senescence and death of roots and can cause a decline in root function. In these cases, roots can only adsorb metals on their surface without transporting them into root tissue cells and translocating them into shoots.

Another reaction of the grass's roots to Cd treatment was an increased release of organic compounds to the growth medium and their storage on the root sur-

face. Numerous authors have observed that Cd-stress induces changes in plant metabolism, with plants synthesizing concentrations of organic compounds that are significantly higher than those synthesized by plants growing in unpolluted environments (dell'Amico *et al.*, 2005; Madhaiyan *et al.*, 2007; Adeniji *et al.*, 2010; Kim *et al.*, 2010). Li *et al.* (2014) have reported that exudation of high amounts of dissolved organic carbon by roots of *Triticum aestivum* is an important protective mechanism under Cd stress. In our study, after cultivation of *F. ovina*, the growth medium contaminated with $10 \, \mu g \, Cd \, ml^{-1}$ was significantly enriched with various organic chelators, siderophores, citric acid, reducing sugars and different proteins (Table III). By contrast, the amounts of phenols in the growth medium decreased to zero at this Cd dose. This drop could have been caused by accumulation of soluble phenolics in root cells and their use in the phenylpropanoid (phenyloalanine) pathway connected with induction of plant resistance to different biotic and abiotic stresses (Atkinson and Urwin, 2012; Khan *et al.*, 2015; Zhang *et al.*, 2015). One of the main results of the induction of this pathway is lignin biosynthesis, which leads to straightening of the plant cell wall by its lignification (Schützendüdel *et al.*, 2001; Elobeid *et al.*, 2012). Acceleration of cell wall lignification under Cd-stress can, on the one hand, negatively affect root elongation, but, on the other, increase Cd immobilization and detoxification, and ultimately enhance tolerance of the plants to this metal.

The exposure of sheep's fescue to $10 \, \mu g \, Cd \, ml^{-1}$ also significantly increased the amounts of organic compounds, other than citric acid, attached to the root surface (Fig. 2). A similar increase has been observed for *S. cereale* cultivated in the same conditions (Majewska and Kurek, 2011). The thick layer of organic material developing on the root surface acted as the first barrier against Cd entering into root tissues while organic compounds secreted into the growth medium played an important role in decreasing the concentration of free metal ions. After *F. ovina* cultivation, only part of the Cd introduced into the hydroponic solution remained in the medium in the ionic form (Table II).

Non-sterilized grass seeds were used in the experiments. The microbial community of these seeds inhabited the growth medium and the root surface of cultivated plants (Table IV). Proliferation of microorganisms was stimulated in the presence of organic compounds, which were efficiently released by roots of plants growing at $10 \, \mu g \, Cd \, ml^{-1}$. The numbers of bacteria in the growth medium were significantly higher at $10 \, \mu g \, Cd \, ml^{-1}$ than in the control medium without cadmium. Also, the numbers of bacteria attached to the root surface and removed during extraction were the highest at $10 \, \mu g \, Cd \, ml^{-1}$. Fungi were found neither in the growth medium nor on the root surface in either

of the Cd treatments. The numbers of bacteria isolated from *F. ovina* roots after extraction declined with the increase in Cd concentration in the growth medium, and at $10 \, \mu g \, Cd \, ml^{-1}$ their titer was two orders of magnitude lower than at $1 \, \mu g \, Cd \, ml^{-1}$ (Table IV). In the natural environment, plant roots are always associated with microorganisms, which utilize organic compounds released to the root zone and produce their own metabolites. Endophytes (strains inhabiting the internal tissues of plants), such as *Serratia nematodiphila*, *Enterobacter aerogenes*, *Enterobacter* sp. or *Acinetobacter* sp. (Chen *et al.*, 2010), and rhizosphere bacteria, such as those belonging to the genera *Agrobacterium*, *Arthrobacter*, *Mycobacterium*, *Pseudomonas* (dell'Amico *et al.*, 2005), *Burkholderia* and *Methylobacterium* (Madhaiyan *et al.*, 2007), could protect plants from the toxic effect of metals (biosorption and precipitation), produce plant growth-promoting compounds (indole acetic acid, siderophores and ACC-deaminase) and improve Fe and P nutrition. In contaminated habitats, some sensitive microbes would be eliminated while others would develop resistance to heavy metals and proliferate. Kao *et al.* (2006), for example, have demonstrated that microorganisms adapted to high Zn, Cu and Pb concentrations in soil, and that their respiration, expressed as CO_2 production, strongly increased within 7 days after metal supplementation.

Cadmium applied at the concentrations of 1 and $10 \, \mu g \, ml^{-1}$ of the growth medium was immobilized by *F. ovina* roots in different amounts (1.7 and $5.4 \, mg \, g^{-1}$, respectively). Roots are composed of many different compounds which are responsible for immobilization of metals. Chen *et al.* (2013) have suggested that pectin and cellulose, but not hemicellulose, present in the cell walls of *Salix jiangsuensis J172* were responsible for Cd adsorption. Next to cellulose, the most abundant natural raw material is lignin. Lignin is an aromatic polymer which contains several functional groups, *e.g.* methoxyl, aliphatic and phenolic hydroxyl, and carboxyl groups, that can act as very efficient binding agents in the immobilization of heavy metal ions (Šćiban *et al.*, 2011). The results of the present study revealed that 43% of Cd immobilized by the roots of plants grown at both cadmium concentrations was retained in their tissues and not released during sequential extraction (Fig. 1). Yin *et al.* (2015), in a study of *Boehmeria nivea*, have recorded the highest concentration of Cd in the cytoderm fraction (precipitated Cd or Cd bound in cell walls), which was found to be the most stable fraction of the metal in plant tissues. This metal could be mobilized only after cell lysis and microbial mineralization of dead tissues. The remaining part of Cd immobilized by *F. ovina* roots was loosely bound (deionized water extractable metal), exchangeably bound (extracted with $0.1 \, M \, NaNO_3$) and bound by chelation (Cd extracted

with 0.02 M EDTA). Cadmium mobilized by deionized water and 0.1 M $NaNO_3$ solution with an ionic strength equal to the strength of soil solution is considered to be the most labile. Cadmium extracted with EDTA is strongly bound and difficult to remove by soil solution (Kurek and Majewska, 2004). The increase in Cd concentration in the medium from 1 to 10 µg ml^{-1} resulted in elevated amounts of Cd extracted from roots with deionized water and 0.1 M $NaNO_3$, and decreased amounts of Cd extracted with 0.02 M EDTA (Fig. 1B), which means that the stability of Cd immobilized by roots was weakened.

Cadmium extracted from *F. ovina* roots was released as free ions and as metal bound to soluble organic compounds. Metal-organic complexes present in the root zone have different degrees of toxicity, bioavailability and degradation. It has been proven that the rate of mineralization of metal-organic complexes such as Cd-acetate and Cd-citrate was significantly lower than for complexes with Mg or Zn (Renella *et al.*, 2004). In such cases, metal-organic complexes can be incorporated into the soil organic matter during humification and humus accumulation. In the natural environment, microbiological decomposition of plant residues is a source of substrates for the synthesis of macromolecular polymers that are combined into aggregates of humic substances (Semenov *et al.*, 2013). If grass shoots with a low Cd concentration (~ 0.76 µg mg^{-1} DW) return to the soil as plant residues, they could facilitate humification and efficiently increase metal stabilization in re-vegetated soils. Further studies are required, however, to fully understand the mechanisms of HM phytostabilization in contaminated soils.

Conclusion

In order to exclude Cd retention by soil solid components, hydroponic cultures were chosen for the experiments. The results of the present study show that Cd-stress decreased *F. ovina* biomass, shoot height, root length and root activity but increased the quantity of root exudates, especially at 10 µg ml^{-1} of the growth medium. At this Cd concentration, root exudates containing organic chelators, siderophores, proteins and reducing sugars significantly stimulated proliferation of bacteria introduced into the hydroponic solution with non-sterilized seeds. Fungi were not detected.

F. ovina immobilized Cd in roots (BAF = 539), with a very low translocation of this metal to shoots (TRL = 0.14). Therefore, this grass could be potentially used for phytostabilization of metals in contaminated soils. Cadmium immobilized by roots was released during sequential extraction as free ions or in a form

bound with organic compounds. These observations suggest that the dissolution, ion exchange and chelation processes occurring in the close vicinity of roots can release Cd immobilized in those roots. The root zone is a specific habitat created by root exudates and microorganisms, and mechanisms controlling Cd phytostabilization are difficult to determine precisely due to the multiple factors acting in this zone.

Literature

Adeniji B.A., M.T. Budimir-Hussey and S.M. Macfie. 2010. Production of organic acids and adsorption of Cd on roots of durum wheat (*Triticum turgidum* L. var. *durum*). *Acta Physiol. Plant.* 32: 1063–1072.

Alef K. and P. Nannipieri. 1995. Methods in applied soil microbiology and biotechnology. Academic Press, London.

Alvarenga P., A.P. Gonçalves, R.M. Fernandes, A. de Varennes, G. Vallini, E. Duarte and A.C. Cunha-Queda. 2008. Evaluation of composts and liming materials in the phytostabilization of a mine soil using perennial ryegrass. *Sci. Total Environ.* 406: 43–56.

An L., Y. Pan, Z. Wang and C. Zhu. 2011. Heavy metal absorption status of five plant species in monoculture and intercropping. *Plant Soil* 345: 237–245.

Arnow L.E. 1937. Colorimetric determination of the components of 3,4-dihydroksyphenylalanine-tyrosine mixtures. *J. Biol. Chem.* 228: 531–537.

Atkin C.L., J.B. Neilands and H. Phaff. 1970. Rhodotorulic acid from species of *Rhodospirillum*, *Rhodotorula*, *Sporidiobolus* and *Sporobolomyces*. *J. Bacteriol.* 103: 722–733.

Atkinson N.J. and P.E. Urwin. 2012. The interaction of plant biotic and abiotic stresses: from genes to the field. *J. Exp. Bot.* 63(10): 3523–3544.

Atlas R.M. 1995. Handbook Media for Environmental Microbiology. CRC Press, Boca Raton.

Bradford M.M. 1976. A rapid and sensitive method for quantification of microgram quantities of proteins utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254.

Brandt S. 1999. Date analysis. Statistical and computational methods for scientist and engineers, 3th ed. Springer-Verlang, New York.

Chen G., Y. Liu, R. Wang, J. Zhang and G. Owens. 2013. Cadmium adsorption by willow root: the role of cell walls and their subfractions. *Environ. Sci. Pollut. Res.* 20: 5665–5672.

Chen L., S. Luo, X. Xiao, H. Guo, J. Chen, Y. Wan, B. Li, T. Xu, Q. Xi, C. Rao and others. 2010. Application of plant growth-promoting endophytes (PGPE) isolated from *Solanum nigrum* L. for phytoextraction of Cd-polluted soils. *Appl. Soil Ecol.* 46: 383–389.

Cheraghi M., B. Lorestani, N. Khorasani, N. Yusefi and M. Karami. 2011. Finding on the phytoextraction and phytostabilization of soils contaminated with heavy metals. *Biol. Trace Elem. Res.* 144: 1133–1141.

Comas L.H., D.M. Eissenstat and A.N. Lakso. 2000. Assessing root death and root system dynamic in a study of grape canopy pruning. *New Phytol.* 47: 171–178.

Csaky T.Z. 1948. On the estimation of bound hydroxylamine in biological materials. *Acta Chem. Scand.* 2: 370–386.

de Ascencao A.R.F.D.C. and I.A. Dubery. 2003. Soluble and wall-bound phenolics and phenolic polymers in *Musa acuminataroots* exposed to elicitors from *Fusarium oxysporum* f.sp. *cubense*. *Phytochemistry* 63: 679–686.

dell'Amico E., L. Cavalca and V. Andreoni. 2005. Analysis of rhizobacterial communities in perennial *Gramicaceae* from polluted water meadow soil, and screening of metal-resistant, potentially plant growth-promoting bacteria. *FEMS Microbiol. Ecol.* 52: 153–162.

Elobeid M., C., Göbel, I. Feussner and A. Polle. 2012. Cadmium interferens with auxin physiology and lignification in poplar. *J. Exp. Bot.* 63(3): 1413–1421.

Fiedler H.D., J.L. Westrup, A.J. Souza, A.D. Pavei, C.U. Chagas and F. Nome. 2004. Cd (II) determination in the presence of aqueous micellar solutions. *Talanta* 64: 190–195.

Ganesan V. 2012. Rhizoremediation: a pragmatic approach for remediation of heavy metal-contaminated soil, pp. 147–162. In: Zaidi A., P.A. Wani and M.S. Khan (eds). Toxicity of heavy metals to legumes and bioremediation. Springer-Verlag Wien.

Gerhardt K.E., X.-D. Huang, B.R. Glick and B.M. Greenberg. 2009. Phytoremediation and rhizoremediation of organic soil contaminants: Potential and challenges. *Plant Sci.* 176: 20–30.

Grodzińska K. and G. Szarek-Łukaszewska. 2009. Heavy metal vegetation in the Olkusz region (Southern Poland) – preliminary studies. *Pol. Bot. J.* 54(1): 105–112.

Haoliang L., Y. Chongling and L. Jingchum. 2007. Low-molecular-weight organic acids exuded by mangrove (*Kandelia candel* (L.) Druce) roots and their effect on cadmium species change in the rhizosphere. *Environ. Exp. Bot.* 61: 159–166.

Huang P.M. and J.J. Germina. 2002. Chemical and biological processes in the rhizosphere: metal pollutants, pp. 381–438. In: Huang P.M., J.-M. Bollag and N. Senesi (eds). Interaction between soil particle and microorganisms. Impact on the terrestrial ecosystem. IUPAC series on analytical and physical chemistry of environmental systems, vol. 8. John Wiley & Sons LTD, Chichester.

Jha P.N., G. Gupta, P. Jha and R. Mehrotra. 2013. Association of rhizospheric/endophytic bacteria with plants: a potential gateway to sustainable agriculture. *Greener J. Agric. Sci.* 3(2): 73–84.

Kacálková L., P. Tlustoš and J. Száková. 2014. Chromium, nickel, cadmium, and lead accumulation in maize, sunflower, willow, and poplar. *Pol. J. Environ. Stud.* 23 (3): 753–761.

Kamaludeen S.P.B. and K. Ramasamy. 2008. Rhizoremediation of metals: harnessing microbial communities. *Indian J. Microbiol.* 48: 80–88.

Kao P.-H., C.-C. Huang and Z.-Y. Hseu. 2006. Response of microbial activities to heavy metals in a neutral loamy soil treated with biosolid. *Chemosphere* 64: 63–70.

Khan M.I.R., M. Fatma, T.S. Per, N.A. Anjum and N.A. Khan. 2015. Salicylic acid-induced abiotic stress tolerance and underlying mechanisms in plants. *Front. Plant Sci.* 6: 462–478.

Kim K.-R., G. Owens, R. Naidu and S.-I. Kwon. 2010. Influence of plant roots on rhizosphere soil solution composition of long-term contaminated soils. *Geoderma* 155: 86–92.

Kurek E. and M. Majewska. 2004. *In vitro* remobilization of Cd immobilized by fungal biomass. *Geoderma* 122: 235–246.

Kurek E. and M. Majewska. 2012. Microbially mediated transformations of heavy metals in rhizosphere, pp. 129–146. In: Zaidi A., P.A. Wani and M.S. Khan (eds). Toxicity of heavy metals to legumes and bioremediation, Springer-Verlag Wien.

Li Y., L. Wang, L. Yang and H. Li. 2014. Dynamics of rhizosphere properties and antioxidative responses in wheat (*Triticum aestivum* L.) under cadmium stress. *Ecotox. Environ. Safe* 102: 55–61.

Lou Y., H. Luo, T. Hu, H. Li and J. Fu. 2013. Toxic effects, uptake, and translocation of Cd and Pb in perennial ryegrass. *Ecotoxicology* 22: 207–214.

Madhaiyan M., S. Poonguzhali and S. Tongmin. 2007. Metal tolerating methylotrophic bacteria reduces nickel and cadmium toxicity and promotes plant growth of tomato (*Lycopersicon esculentum* L.). *Chemosphere* 69: 220–228.

Majewska M., E. Kurek and D. Szlachetka. 2006. Microbial activity – factor increasing retention of Cd added to soil. *Pol. J. Environ. Stud.* 15(2a): 127–134.

Majewska M. and E. Kurek. 2011. Effect of Cd concentration in growth media on *Secale cereale* roots and Cd interaction with rhizosphere microorganisms originating from different parts of the grain. *Eur. J. Soil. Biol.* 47: 95–101.

Majewska M., E. Kurek and A. Słomka. 2011. 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. *Pol. J. Environ. Stud.* 20(3): 591–297.

Martin J.P. 1950. Use of acid, rose bengal and streptomycin in the plate method for estimating soil fungi. *Soil Sci.* 38: 215–220.

Mendez M.O. and R.M. Maier. 2008. Phytostabilization of mine tailing in arid and semiarid environments – an emerging remediation technology. *Environ. Health Persp.* 116(3): 278–283.

Philippot L., J.M. Raaijmakers, P. Lemanceau and W.H. van der Putten. 2013. Going back to the roots: the microbial ecology of the rhizosphere. *Nat. Rev. Microbiol.* 11: 789–799.

Renella G., L. Landi and P. Nannipieri. 2004. Degradation of low molecular weight organic acids complexed with heavy metals in soil. *Geoderma* 122: 311–315.

Schützendübel A., P. Schwanz, T. Teichmann, K. Gross, R. Langenfeld-Heyser, D.L. Godbold and A. Polle. 2001. Cadmium-induced changes in antioxidative systems, hydrogen peroxide content, and differentiation in scots pine roots. *Plant Physiol.* 127: 887–898.

Šćiban M.B., M.T. Klašnja and M.G. Antov. 2011. Study of the biosorption of different heavy metal ions onto Kraft lignin. *Ecol. Eng.* 37: 2092–2095.

Semenov V.M., A.S. Tulina, N.A. Semenova and L.A. Ivannikova. 2013. Humification and nonhumification pathways of the organic matter stabilization in soil: A review. *Eurasian Soil Sci.* 46(4): 355–368.

Sipos G., Á. Solti, V. Czech, I. Vashegyi, B. Tóth, E. Cseh and F. Fodor. 2013. Heavy metals accumulation and tolerance of energy grass (*Elymus elongates* subsp. *ponticus* cv. Szavasi-1) grown in hydroponic culture. *Plant Physiol. Bioch.* 68: 96–103.

Soleimani M., H.A. Hajabbasi, M. Afyuni, A. Mirlohi, O.K. Borggaard and P.E. Holm. 2010. Effect of endophytic fungi on cadmium tolerance and bioaccumulation by *Festuca arundinacea* and *Festuca pratensis*. *Int. J. Phytoremediat.* 12: 535–549.

Van der Perk M. 2006. Soil and Water Contamination from Molecular to Catchment Scale. Taylor & Francis/Balkema, Leiden.

Xu Y. and F.M.M. Morel. 2013. Cadmium in Marine Phytoplankton, pp. 509–528. In: Sigiel A., Sigiel, H. and R.K.O. Sigiel, (eds). Cadmium: From Toxicity to Essentiality, Metal Ions in Life Sciences. Springer Sciences + Business Media, Dordrecht.

Yin Y., Y. Wang, Y. Liu, G. Zeng, X. Hu, X. Hu, L. Zhou, Y. Guo and J. Li. 2015. Cadmium accumulation and apoplastic and symplastic transport in *Boehmeria nivea* (L.) *Gaudich* on cadmium-contaminated soil with the addition of EDTA or NTA. *RSC Advances* 5: 47584–47591.

Zhang S., T. Li, H. Huang, Z. Zou, X. Zhang, H. Yu, Z. Zheng and Y. Wang. 2012. Cd accumulation and phytostabilization potential of dominant plants surrounding mining tailings. *Environ. Sci. Pollut. Res.* 19: 3879–3888.

Zhang Y., S. Xu, S. Yang and Y. Chen. 2015. Salicylic acid alleviates cadmium-induced inhibition of growth and photosynthesis through upregulating antioxidant defense system in two melon cultivars (*Cucumis melo* L.). *Protoplasma* 252: 911–924.