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Abstract: Phytoremediation is a plant based, cost effective technology to detoxify or stabilize contaminated soils. Fast growing, high biomass, perennial plants may be used not only in phytoremediation but also in energy production. Szarvasi-1 energy grass (Elymus elongatus subsp. ponticus cv. Szarvasi-1), a good candidate for this combined application, was grown in nutrient solution in order to assess its Cd, Cu, Ni, Pb and Zn accumulation and tolerance. Its shoot metal accumulation showed the order Pb<Ni<Cu~Cd<Zn. In parallel with this, Pb and Ni had no or very little influence on the growth, dry matter content, chlorophyll concentration and transpiration of the plants. Cu and Cd treatment resulted in significant decreases in all these parameters that can be attributed to Fe plaque formation in the roots suggested by markedly increased Fe and Cu accumulation. This came together with decreased shoot and root Mn concentrations in both treatments while shoot Cu and Zn concentrations decreased under Cd and Cu exposure, respectively. Zn treatment had no effect or even slightly stimulated the plants. This may be due to a slight stimulation of Fe translocation and a very efficient detoxification mechanism. Based on the average 300 mg kg-1 (dry mass) Zn concentration which is 0.03% of the shoot dry mass the variety is suggested to be classified as Zn accumulator.

To the Editor-In-Chief of Plant Physiology and Biochemistry

Dear Mario,

I am sending the manuscript entitled „Heavy metal accumulation and tolerance of energy grass (Elymus elongatus subsp. ponticus cv. Szarvasi-1) grown in hydroponic culture" by Gyula Sipos et. al. for publication in Plant Physiology and Biochemistry. The manuscript has been prepared on the basis of the latest instructions to authors.

The manuscript deals with an economically important plant variety now applied in Europe and Asia for renewable energy production. However, the plant has a predicted capacity to scavange high concentrations of toxic or essential metals from contaminated soils due to its high tolerance to stresses, fast growing habit and large biomass. So far, there is no detailed study to assess the metal accumulation as well as its physiological background in this plant.

The research highlights of our work are the following:

1. Shoot metal accumulation in Szarvasi-1 energy grass showed the order Pb<Ni<Cu~Cd<Zn.

2. Pb and Ni had no or very little while Cu and Cd had negative effect on the physiological parameters (growth, dry matter content, chlorophyll concentration and transpiration).

3. Cu and Cd effect can be attributed to Fe plaque formation in the roots suggested by increased Fe and Cu accumulation. This came together with an imbalance in shoot microelement (Mn, Cu, Zn) levels.

4. Szarvasi-1 energy grass proved to be a Zn accumulator plant with 300 mg kg^{-1} (dry mass) shoot Zn concentrations.

5. Zn increased Chl concentration and transpiration which may be due to a slight stimulation of root to shoot Fe translocation and a very efficient detoxification mechanism.

I hope that the manuscript is suitable for publication in PPB.

With best regards,

Ferenc Fodor

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*Highlights (for review)

1. Shoot metal accumulation in Szarvasi-1 energy grass showed the order Pb<Ni<Cu~Cd<Zn.

2. Szarvasi-1 is a Zn accumulator plant with 300 mg kg^{-1} DW Zn in shoots.

3. Zn increased shoot Fe and Chl concentrations and transpiration.

4. Cu and Cd reduced growth, Chl concentration and transpiration.

5. Cu and Cd caused Fe plaque formation in roots and microelement imbalance in shoots.

Heavy metal accumulation and tolerance of energy grass (*Elymus elongatus* subsp. *ponticus* cv. Szarvasi-1) grown in hydroponic culture

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Abstract

Phytoremediation is a plant based, cost effective technology to detoxify or stabilize contaminated soils. Fast growing, high biomass, perennial plants may be used not only in phytoremediation but also in energy production. Szarvasi-1 energy grass (*Elymus elongatus* subsp. *ponticus* cv. Szarvasi-1), a good candidate for this combined application, was grown in nutrient solution in order to assess its Cd, Cu, Ni, Pb and Zn accumulation and tolerance. Its shoot metal accumulation showed the order Pb<Ni<Cu~Cd<Zn. In parallel with this, Pb and Ni had no or very little influence on the growth, dry matter content, chlorophyll concentration and transpiration of the plants. Cu and Cd treatment resulted in significant decreases in all these parameters that can be attributed to Fe plaque formation in the roots suggested by markedly increased Fe and Cu accumulation. This came together with decreased shoot and root Mn concentrations in both treatments while shoot Cu and Zn concentrations decreased under Cd and Cu exposure, respectively. Zn treatment had no effect or even slightly stimulated the plants. This may be due to a slight stimulation of Fe translocation and a very efficient detoxification mechanism. Based on the average 300 mg kg^{-1} (dry mass) Zn concentration which is 0.03% of the shoot dry mass the variety is suggested to be classified as Zn accumulator.

Key words: heavy metal accumulation; iron plaque; phytoremediation; Szarvasi-1 energy grass; tall wheatgrass; zink accumulator

1. Introduction

Heavy metal contamination in soils is a worldwide environmental problem. The contamination may be originated from natural and anthropogenic sources, the latter being much more significant. Anthropogenic contamination may occur due to mining, industrial activities, traffic, inadequate use of (phosphate) fertilisers in agriculture and amendment with sewage sludge [1]. Heavy metals, naturally present or deposited in various concentrations, have different solubility and mobility in the soil but may be mobilised and accumulated by plants [2]. This means a major threat for heavy metal uptake by crop plants but also provide a possibility to remove the metals from the soils by specific plant species. Phytoremediation techniques based on naturally metal accumulating plants (accumulator plants) or chelate-assisted metal mobilization and uptake, i.e. phytoextraction [3,4], may raise another problem of the fate of harvested plant material. Fast growing, high biomass, perennial plants developed or genetically designed for energy production may provide a feasible and cost-effective solution [5].

Szarvasi-1 energy grass (*Elymus elongatus* subsp. *ponticus* cv. Szarvasi-1) was bred from a native population of tall wheatgrass in Hungary that was adapted to slightly salty habitats [6,7]. It has a fibrous root system that may reach 3.5 m whereas the shoot may grow to 1.8-2.2 m. In spite of its high biomass yield it is well adapted to drought, flood and frost and does not require special soil conditions but prefers sandy and alkaline soils. As a perennial grass it may live up to 10-15 years. Its industrial uses are well documented but there are only limited data available on its natural element composition or requirement and accumulation [8].

Heavy metals, such as cadmium, copper, lead, mercury, nickel and zinc are major pollutants, particularly in areas with high anthropogenic pressure [9]. Szarvasi-1 energy grass may be a potentially applied in renewable energy production combined with phytoextraction or phytostabilization. The aim of the present work was to assess the natural ability of Szarvasi-1 energy grass to accumulate or tolerate different heavy metals, Cd, Cu, Ni, Pb, Zn from nutrient solution. Hydroponic culture was chosen for the experiments because it excludes the different adsorption, mobility and retention characteristics of the metals in soil.

2. Results

2.1. Physiological responses to heavy metal treatments

The control and heavy metal containing nutrient solutions had very similar, slightly acidic pH values which have been increased to slightly alcaline levels during cultivation of the plants (Table 1). The extent of increase was smaller in case of Cd and Cu treatments.

Root and shoot growth was not affected, compared to the untreated, control by Pb and Zn applied in the nutrient solution in 10 μM concentration for a month (Fig. 1). Ni and Cd caused about 20 and 35% inhibition in the root and shoot growth, respectively. Szarvasi-1 was the most sensitive to Cu that decreased the root and shoot dry mass by 90 and 75%, respectively. When Cd and Cu was applied the relative dry matter content of the roots increased with 76 and 138% whereas that of the shoots with 44 and 56%, respectively (Fig. 2).

The Chl concentration of the leaves changed most markedly under Cu treatment that caused about 50% decrease leading to visible symptoms (Fig.3). Cd and Ni caused a smaller but significant decrease compared to the control while the effect of Pb was insignificant. The transpiration, measured as stomatal conductance for water vapour, decreased by 79 and 91% in the plants treated with Cd and Cu, respectively (Fig. 4). Zn increased the Chl concentration and stimulated the transpiration compared to the control, although these changes were not significant.

2.2. Heavy metal concentration

Heavy metals applied in the treatments in 10 μM concentration were adsorbed by the roots in different amounts (Fig. 5). Cd and Ni concentrations were very similar and the lowest among the five metals. Zn concentration was twice as large while Cu concentration was almost 4 times larger than that of Cd and Ni. Pb was adsorbed in the highest amount (280 $\mu mol\ g^{-1}$ DW=1.35 $mg\ kg^{-1}$ DW). Half of the roots of each plant were undertaken a washing procedure (with $CaSO_4$ +Na_2EDTA) in order to remove the loosely bound part of the adsorbed metals. The metal concentration was recalculated using the same dry mass data. The results revealed that most of the Cd (79%) and Ni (93%) were not removable. In case of Zn only 41% remained in the roots after washing. However, the root concentrations of the three metals were statistically not different after the washing (26-27 $\mu mol\ g^{-1}$ DW). In case of Cu and Pb most of the adsorbed amount was removed in the washing procedure: only 8 and 1% remained, respectively, resulting in the lowest concentrations.

In the shoot, Cd and Cu concentrations were similar while Ni and Pb were significantly lower. Zn concentration was the highest reaching 300 $mg\ kg^{-1}$ DW which is 0.03% of the shoot dry mass.

2.3. Essential metal concentration

The concentration of Fe was similar in the roots and shoots of control, Ni, Pb and Zn treated plants (Fig. 6). However great difference was found between the Cd and Cu treated plants.

The latter two treatments caused a very high increase in the total Fe concentration of the roots, 140 and 400 % by the Cd and Cu treatment, respectively, and tThe non-removable fraction was still several times higher than in the control. The shoot concentration was significantly changed (increased) only by Zn. Total Mn concentrations in the roots of Ni and Pb treated plants were the same as in the control (Fig.7). Zn treatment reduced the Mn concentration to one third of the control while Cd and Cu further reduced it to a minimal level. The non-removable fraction of Mn accounted for 33-53% of the total amount. The shoot contained Mn at a similar level in the control, Ni and Pb treatment while Zn slightly reduced it. Cd and Cu decreased the shoot Mn to about half of the control.

Zn and Cu concentrations of the plants were compared in Figs. 8 and 9 when they were applied at low (microelement) concentration. Only the roots of Cd and Ni treated plants contained Zn in significantly lower concentration than the control (Fig.8). However, the non-removable fraction was different from the total only in the control. In the shoot, only the Cu treated plants contained Zn at a lower level. Cu concentrations were almost identical to the control in the Ni, Pb and Zn treatment in both roots and shoots while in the Cd treated plants it was 50% higher in the root and 20% lower in the shoot (Fig.9).

3. Discussion

3.1. Heavy metal uptake

The metal content of shoot tissues depends on the uptake and translocation ability of root and vascular tissues. In the hydroponic culture, the root system of Szarvasi-1 energy grass adsorbed highly different amounts of heavy metals at slightly acidic to slightly alkaline pH in the order: Pb>Cu> Zn> Cd≥Ni (Fig 5.). This finding is in agreement with previous work on tall wheatgrass [10] and may be explained by different mechanisms. Cu, Zn and Ni are essential transition metals required for normal growth in the order Zn>Cu> Ni and are readily soluble in the applied experimental conditions. Their adsorbance may be driven by active uptake. Cd is a nonessential heavy metal that is present in the nutrient solution in free divalent ionic form [11]. However, after applying a washing procedure ($CaSO_4$ + Na_2EDTA solution) in order to remove the portion deposited only to the apoplastic spaces, we found that Cd, Ni and Zn were taken up by the roots in very similar amount, while Cu uptake was smaller. The influx of transition metals is mediated by specific transporter proteins.

Zn uptake was first reported to be regulated by ZIP family genes in *Arabidopsis thaliana* [12,13], however, the exact function of ZIPs is poorly known, yet [14]. In rice, a Strategy II plant in Fe uptake, OsZIP1 and OsZIP3 seems to be important for Zn uptake from soil [15,16]. In barley, Zn-DMA (deoxy mugineic acid – a phytosiderophore released by the plant) is preferred over Zn^{2+} for uptake through roots [17]. In contrast, rice plants absorb less Zn-DMA compared to Zn^{2+} [18]. Tall wheatgrass is a close relative to barley, thus DMA-chelated Zn uptake can be predicted.

Cd may enter the root cells using different pathways provided by ZIP family transporters, ZNT1 [19] and IRT1 which latter mediates Fe^{2+} uptake in non-graminaceous plants [20] but was also found in rice [21]. In rice, Cd^{2+} uptake into the symplasm was shown to be linked to Ca^{2+} transport, as accumulation of Cd is inhibited by La^{3+} and high Ca^{2+} concentrations [22]. Wheat LCT1 (low-affnity cation transporter) was shown to have a role in both Cd^{2+} and Ca^{2+} uptake [23]. In rice, OsNramp5 and OsNramp1 were reported as a root plasma membrane transporter of Mn^{2+} and Cd^{2+} [24] and Fe^{2+} and Cd^{2+} [25], respectively.

The uptake and translocation of Cu is little known, it was found that P-type heavy metal ATPases (HMAs) are involved [26,27]. Cu^{+} transport into the cytosol is also mediated by COPT family transporters in *A. thaliana* [28]. In graminaceous plants, the uptake of Cu (and Zn) may be mediated by the release of phytosiderophores which (is increased under Fe and Zn deficiency and) plays a distinct role in Fe acquisition [29]. ZIP2 and ZIP4 proteins are also suggested to be transporting Cu^{2+} in *Arabidopsis* [30]. Cu^{2+} can form stable NA chelate even under mild acidic condition which complexes may have a role in the Cu translocation. The uptake of Cu^{2+}-chelates cannot be excluded in strategy-II plants, either. Gunawardana et al [31] showed that Cu uptake is enhanced by the presence of hystidine in the hydroponic solution in ryegrass (*Lolium perenne*).

The uptake and translocation of Ni is poorly known, too. Ni may enter the cells in a rather unspecific route through plasmalemma CNGCs (cyclic nucleotide gated channels) [32]. Nishida et al. [33] showed that AtIRT1, the primary Fe^{2+} uptake transporter in the root, mediates Ni accumulation in *Arabidopsis thaliana*. But there is no evidence for a specific Ni uptake in strategy-II plants up to now.

Taking all these into account, ZIP-family transporters or chelation based strategies (NA and DMA chelation) may be involved in the uptake of Cu, Ni and Zn. Cd uptake may also interfere with that of Fe and Mn. Thus, regular disturbances in the essential transition metal uptake and translocation in heavy metal treated Szarvasi-1 energy grass can be explained as complex interference in these systems.

The various uptake mechanisms described above show that probably it is not the way of influx that matters as it does not provide explanation for the higher adsorption and lower uptake of Cu compared to the other transition metals in Szarvasi-1 energy grass. The formation of Fe-plaque in the roots of Cu treated plants may account for the retention of Cu and also Fe on the roots [34]. Such an unspecific mechanism may be predicted in the case of Cd and Pb, too. In Cd treated plants this is underlined by the accumulation of Fe and Cu in the root apoplast. Pb is a nonessential heavy metal that may produce relatively insoluble precipitates with the constituents of the nutrient solution in sulphate and phosphate (or chloride) form on the root surface [35]. Soluble Pb concentration can be increased with complexing agents like EDTA or citrate [8] which serves as the basis for Pb mobilization in polluted soils during “induced phytoextraction” [36,37,38]. Applying the ($CaSO_4$ + Na_2EDTA) washing procedure, we found that Pb taken up by the roots was almost negligible. Pb uptake may be mediated either by CNGC [32] or P-type ATPase transporters [39]. However, as most of this metal is removable from the root apoplast its uptake may occur through more unspecific routes, too. The ionic radius of Pb^{2+} is much larger compared to the other metal ions tested, thus it may be assumed to surge into stelar tissues through internal wounding by lateral root formation [40] or may be taken up by endocytosis [41].

3.2. Heavy metal translocation

The shoot metal concentration in our study increased in the order: Pb<Ni<Cu~Cd<Zn (Fig. 5). This is in contradiction with the finding of Yang et al. [10] even though data were compared after recalculation based on their figures. They found that the accumulation depended on the applied concentration and it increased in the order Pb<Cd<Cu<Ni at 0.5 mM metal dose. The authors applied chloride form of the metals and much higher concentrations than the present work. Although, the accumulation order was different, Pb accumulation in the shoot was the smallest, too. In our previous work, it was found that Pb accumulation in the shoot may even be lower if the Fe-chelator applied in the nutrient solution is EDTA while citrate may be effective in increasing shoot Pb accumulation at higher Pb levels in the medium [8]. The low Pb uptake and accumulation was shown also by the low TI and PC values (Table 2). Once loaded into the xylem, Pb may be transported in Pb-citrate form as it was suggested previously [42].

Nickel transport was so low that hardly exceeded that of Pb in Szarvasi-1 energy grass whereas in Yang et al. [10] it has the highest concentration in the shoot. However, the shoot Ni concentration we found and its translocation (TI=0.077, Table 2) is very similar to that in Chen and Wong [43] (TI=0.1, calculated from the published data) who worked with tall wheatgrass grown in soil. Concerning the mechanism of its translocation, histidine was shown to interfere with the xylem loading of Ni^{2+} [44]. Its transport in the xylem sap was suggested to occur in chelated form [45].

The very low accumulation of Pb and Ni in the shoot implies that their translocation in Szarvasi-1 energy grass is driven only by transpiration and that there is no metabolic demand for Ni, either.

Cu and Cd concentrations in the shoot were very similar in the treated plants and their value was also similar to Fe concentration in all treatments. This may refer to a similar way of xylem loading and/or transport. Xylem loading of Cu and Cd may occur by active efflux through P-type ATP-ases [28,46]. Gunawardana et al. [31] also reported a similar behaviour of Cu and Cd so that additional citric acid enhanced the translocation of both metals. Fe is translocated as ferric-citrate complexes in the xylem sap [47]. Curie et al. [48] showed that Cu-NA complex is completely stable at the pH of xylem sap (pH 5–6) and Cu is transported to the shoot in NA-chelated form. The synthesis of chelators may increase upon Cu excess [49] while the long-distance translocation of Cd, which does not form chelates under *in vivo* conditions, may depend on the availability of other elements [50], and is less dependent on the presence of chelators in the xylem sap. Cu concentration increased 9-fold in the shoot of treated plants compared to the control but this increase came together with severe toxicity symptoms discussed below. This finding is in agreement with previous work [10]. Both Cu and Cd translocation was much higher than that of Pb and Ni and also exceeded that of Fe in the treated plants which latter was highly retarded by the treatments compared to the untreated control (Table 2). However, the PC of Cu and Cd was not considerably higher than that of Pb and Ni and was the same as that of Fe. These findings show that Szarvasi-1 energy grass is not an efficient accumulator of Cd, Cu, Ni and Pb. This is not the case for Zn.

Szarvasi-1 energy grass proved to be very efficient in accumulating Zn in the shoot. Vetiver grass (*Chrysopogon zizanioides*) accumulated 6.2 µmol/g Zn in the shoot (as compared to 4.7 µmol/g in Szarvasi-1 energy grass) but in that case much higher Zn concentrations were measured in the soil solution [37]. Wheat genotypes are different concerning their ability to take up and transport Zn. Zn efficient genotypes release more phytosiderophores which correlates with higher shoot concentrations [51]. Furthermore, Hacisalihoglu et al. [52] identified high and low affinity Zn transport systems in wheat roots, while Zn translocation was shown to be very efficient resulting in balanced concentration in the shoots of radiolabelled plants [53]. Zn-NA transporters or Zn-DMA transporters involved in Zn translocation have not been identified, yet [16] but Zn-NA complexes were shown to exist in the phloem sap of rice [54]. Ishimaru et al. [15] suggested that Zn deficiency induces DMA synthesis in barley shoots, while both Zn and Fe deficiency induce MA synthesis and secretion in barley roots. These data indicate that Strategy-II plants may efficiently scavange Zn from the soil. Although Cd and Zn are chemically very similar [55], they behave differently. These metals showed the highest TI as compared to the other metals in the treatments while the PC values for Zn was 6.5 times higher even than that of Cd (Table 2). This indicates that Szarvasi-1 energy grass is indeed very efficient in Zn accumulation.

3.3. Physiological dysfunctions under heavy metal treaments

The increase in the pH of the nutrient solution under all treatments can be explained by the original habitat preference of tall wheatgrass: it grows in alkaline soils with pH 6-10 (Table

1). The pH increase during cultivation under Cd and Cu treatments was moderate which implies a disturbed metabolism by these metals. This was confirmed by a serious growth inhibition and higher dry matter content whereas neither of the other heavy metals caused any disturbance in these parameters compared to the control except for Ni treatment which led to a slight growth inhibition in the root and shoot (Figs 1, 2).

Interestingly, Pb had no significant effect on any physiological parameters measured in this work. This may have been due to its very low concentration in the root symplast and in the shoot.

Transition metals Cd, Cu and Ni reduced Chl concentration in Szarvasi-1 energy grass (Fig. 3). Cd is known to decrease Chl concentration in Strategy-I plants by decreasing the citrate transporter FRD3 expression in root xylem parenchyma which leads to decreased Fe translocation [56]. Stomatal conductance also decreased under Cd treatment (Fig. 4). Cd is known to interact with Ca metabolism resulting disturbed signalling processes. Thus, the presence of Cd in guard cells leads to stomatal closure [57]. Decreased transpiration rate enhances the inhibition of growth as well as metal translocation (see the decrease in the shoot concentration of Cu and Mn, Figs. 7 and 9).

Ni reduced Chl concentration similary to Cd but its effect on growth was much less pronounced. As it did not modify the uptake and translocation of essential elements and the transpiration, either, it may have reduced only the photosynthetic performance of plants through inhibition of Chl synthesis.

Cu decreased the Chl concentration in the plants to the highest extent which is combined with a significant inhibition in the stomatal conductance. It decreased the shoot Zn and Mn concentration compared to the control (Figs. 7, 8). As Cu did not significantly interfere with Fe translocation to the shoot (Fig. 6), the inhibition of Chl synthesis may have been coupled to the inhibited development of the photosynthetic appartus due to Mn deficiency and a superimposed oxidative stress due to high Cu and low Zn concentration [58]. The lower Chl concentration may have resulted in lower photosynthetic performance that required a lower gas exchange rate leading to stomatal closure.

Zn slightly enhanced the Chl accumulation as well as transpiration (Figs. 3, 4). The uptake of Zn may interact with Fe uptake resulting in higher non-removable Fe concentration in the root and also higher shoot Fe concentration compared to the control (Fig. 6) that may explain the positive effect on the mentioned parameters. Zn transport in the xylem is most probably independent from that of Fe but there is a clear stimulation of Fe translocation along with an inhibition of Mn translocation (Fig. 7) under Zn treatment. In fact, the PC calculated for Fe under Zn treatment was higher than in the control (Table 2). Zn stress is known to inhibit the growth and leaf expansion and may also lead to oxidative stress [59]. However, these effects were not observed in Szarvasi-1 energy grass that implies a very efficient detoxification mechanism in this plant.

4. Conclusions

Szarvasi-1 energy grass, an energy crop potentially applicable in phytoremediation was shown to be sensitive to high external concentrations of Cu and Cd and fairly tolerant to Ni and Pb. Cu and Cd toxicity leading to the inhibition of growth, transpiration and Chl synthesis can be attributed to the uptake and translocation of these metals which in turn causes imbalance in microelement homeostasis and most probably oxidative stress. Fe plaque formation suggested by Fe and Cu accumulation in the roots may also explain the negative effects. Tolerance to Ni and Pb can be explained by the very low translocation of these metals to the shoot due to the unspecific nature of their uptake and transport. Szarvasi-1 showed high rates of Zn translocation to the shoot as compared to the other metals that was combined with high tolerance. Exposure to high concentration of Zn even resulted in a slight stimulation of growth, transpiration and Chl synthesis in paralel with its accummulation in the shoot. Based on these observations the plant is eligible for phytostabilization of Ni-Pb-Zn contaminated soils that has to be confirmed with soil based experiments.

5. Materials and Methods

5.1. Plant material and treatments

The seeds of a tall wheatgrass cultivar, Szarvasi-1 energy grass (*Elymus elongatus* subsp. *ponticus* (Podp.) Melderis cv. Szarvasi-1 (syn. *Agropyron elongatum*, *Elytrigia elongata*, Csete et al., 2011), developed for industrial purposes were germinated for five days on wet filter papers in Petri dishes at room temperature and sunlight. Ten seedlings with 2-5 cm long roots were placed on a 2 cm wide strip of sponge-rubber, rolled up and fastened in a polystyrene ring and they were transferred to plastic containers. Each container was filled up with 0.7 dm^3 modified quarter strength Hoagland nutrient solution of the following composition: 1.25 mM KNO_3; 1.25 mM $Ca(NO_3)_2$; 0.5 mM $MgSO_4$; 0.25 mM KH_2PO_4; 11.6 µM H_3BO_3; 4.5 µM $MnCl_2.4H_2O$; 0.19 µM $ZnSO_4.7H_2O$; 0.12 µM $Na_2MoO_4.2H_2O$; 0.08 µM $CuSO_4.5H_2O$ and 10 µM Fe(III)-citrate-hydrate. Metals were added as $Cd(NO_3)_2$, $CuSO_4.5H_2O$, $NiSO_4$, ZnSO4.7H_2O, $Pb(NO_3)_2$ in 0 or 10 µM concentration to the nutrient solution, separately. Fresh solutions were used for cultivation without buffering or other pH adjustment. The plants modified the original pH of the solutions at the same level during the period between solution changes (Table 1).

The plants were grown in a climate controlled growth chamber at 20/25 ºC, at 75% relative humidity and 150 µmol m^{-2} s^{-1} PPFD with 10/14h dark/light period. The nutrient solution was continuously aerated and replaced fresh solution twice a week.

Three parallel pots, each containing 10 plants were applied for a treatment group ending up in 18 pots with the untreated control and the whole experiment was carried out twice. Physiological parameters were measured and the plants were harvested 37 days after germination.

5.2. Mass measurements

The roots of the 10 plants grown in a single pot were separated into two portions. The roots of the first 5 plants were centrifuged between filter papers at 300 g to remove traces of nutrient solution before drying but no other treatment was applied. The other 5 roots were rinsed with 0.5 mM $CaSO_4$ solution and then transferred to 200 ml 0.5 mM $CaSO_4$ solution containing 10 mM Na_2EDTA (pH 4.05) and were shaken for 1.5 h at 125 rpm [11]. After rinsing again with $CaSO_4$ the roots were centrifuged between filter papers at 300 g. The filtered roots were weighed. Dry mass was determined after drying at 80 °C. Final data are extrapolated to one single plant.

5.3. Element analysis

Measurements were made with dried samples of 5 plants in three parallel after acidic digestion. 5-10 ml $ccHNO_3$ was added to each gram of the samples for overnight incubation. Then the samples were pre-digested for 30 min at 60 °C. Finally, 2-3 ml H_2O_2 (30 m/m%) was added for a 90 min boiling at 120 °C. The solutions were filled up to 10-50 ml, homogenised and filtered through MN 640W filter paper. The element content of the filtrate was determined by ICP-MS. Data were converted from ppm to μmol g^{-1} units in order to ensure better comparison between treatments.

5.4. Chlorophyll concentration

The measurements were made with the first fully developed leaves. The chlorophyll (Chl) concentration was determined photometrically (Shimadzu UV-2101PC) from 80% acetone extracts using the equations of Porra et al. [60]. Each measurement was carried out on three individual plants in each treatment group.

5.5. Stomatal conductance

Stomatal conductance was measured with a porometer (DELTA-T Devices Ltd.) on the abaxial epidermis of the middle sections of the youngest, fully developed leaves. Transpiration was calculated as mmol H_2O m^{-2} s^{-1}. Each measurement was carried out three times on three individual plants in each treatment group.

5.6. Definition of indices

Translocation index (TI) and phytoextraction capacity (PC) was defined after Vashegyi et al. [8] with slight modification. Translocation index of Me_i = shoot total Me_i content (g) / total Me_i content in the washed roots (g). Phytoextraction capacity of Me_i = shoot total Me_i content (g) * 100 / total amount of Me_i supplied to the nutrient solution during the entire growth period (g)

5.7. Statistics

Basic statistical analysis was carried out with one-way ANOVA and Tukey-Kramer multiple comparisons test ($p<0.05$) using Statistica 2000 (Statsoft) and InStat 3.0 (GraphPad) softwares.

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Captions to Figures

Figure 1 Root and shoot dry mass of 37 day-old Szarvasi-1 energy grass grown in nutrient solutions amended with different metals (Cd, Cu, Ni, Pb, Zn) in 0 or 10 μM concentration. (Data are shown as mean±SD, n=6, significant differences between data are idicated with different letters, $P<0.05$)

Figure 2 Dry matter content in the roots and shoots of 37 day-old Szarvasi-1 energy grass grown in nutrient solutions amended with different metals (Cd, Cu, Ni, Pb, Zn) in 0 or 10 μM concentration. (Data are shown as mean±SD, n=6, significant differences between data are idicated with different letters, $P<0.05$)

Figure 3 Chlorophyll concentration in the leaves of 37 day-old Szarvasi-1 energy grass grown in nutrient solutions amended with different metals (Cd, Cu, Ni, Pb, Zn) in 0 or 10 μM concentration. (Data are shown as mean±SD, n=6, significant differences between data are idicated with different letters, $P<0.05$)

Figure 4 Transpiration of the leaves of 37 day-old Szarvasi-1 energy grass grown in nutrient solutions amended with different metals (Cd, Cu, Ni, Pb, Zn) in 0 or 10 μM concentration, measured as the stomatal conductance for water vapour. (Data are shown as mean±SD, n=9, significant differences between data are idicated with different letters, $P<0.05$)

Figure 5 Heavy metal concentration in the roots and shoots of 37 day-old Szarvasi-1 energy grass grown in nutrient solutions amended with the different metals (Cd, Cu, Ni, Pb, Zn) in 0 or 10 μM concentration. Shaded parts of the coloumns show the concentration of metals non-removable by washing with $CaSO_4$+Na_2EDTA solution. (Data are shown as mean±SD, n=6, significant differences between data are idicated with different letters, $P<0.05$)

Figure 6 Fe concentration in the roots and shoots of 37 day-old Szarvasi-1 energy grass grown in nutrient solutions amended with different metals (Cd, Cu, Ni, Pb, Zn) in 0 or 10 μM concentration. Shaded parts of the coloumns show the concentration of Fe non-removable by washing with $CaSO_4$+Na_2EDTA solution. (Data are shown as mean±SD, n=6, significant differences between data are idicated with different letters, $P<0.05$)

Figure 7 Mn concentration in the roots and shoots of 37 day-old Szarvasi-1 energy grass grown in nutrient solutions amended with different metals (Cd, Cu, Ni, Pb, Zn) in 0 or 10 μM concentration. Shaded parts of the coloumns show the concentration of Mn non-removable by washing with $CaSO_4$+Na_2EDTA solution. (Data are shown as mean±SD, n=6, significant differences between data are idicated with different letters, $P<0.05$)

Figure 8 Zn concentration in the roots and shoots of 37 day-old Szarvasi-1 energy grass grown in nutrient solutions amended with different metals (Cd, Cu, Ni, Pb) in 0 or 10 μM concentration. Shaded parts of the coloumns show the concentration of Zn non-removable by washing with $CaSO_4$+Na_2EDTA solution. (Data are shown as mean±SD, n=6, significant differences between data are idicated with different letters, $P<0.05$)

Figure 9 Cu concentration in the roots and shoots of 37 day-old Szarvasi-1 energy grass grown in nutrient solutions amended with different metals (Cd, Ni, Pb, Zn) in 0 or 10 μM concentration. Shaded parts of the coloumns show the concentration of Cu non-removable by washing with $CaSO_4$+Na_2EDTA solution. (Data are shown as mean±SD, n=6, significant differences between data are idicated with different letters, $P<0.05$)

Table 1

Table 1. pH values of the nutrient solutions amended with different heavy metals (Cd, Cu, Ni, Pb, Zn) in 0 (ctr) or 10 μM concentration at preparation (Day 0) and after 4 days of plant growth (Day 4) in unbuffered, aerated hydroponic culture of one month-old Szarvasi-1 energy grass. (Data are presented as mean±SD, n=6, significant differences between data are idicated with different letters, P<0.05))

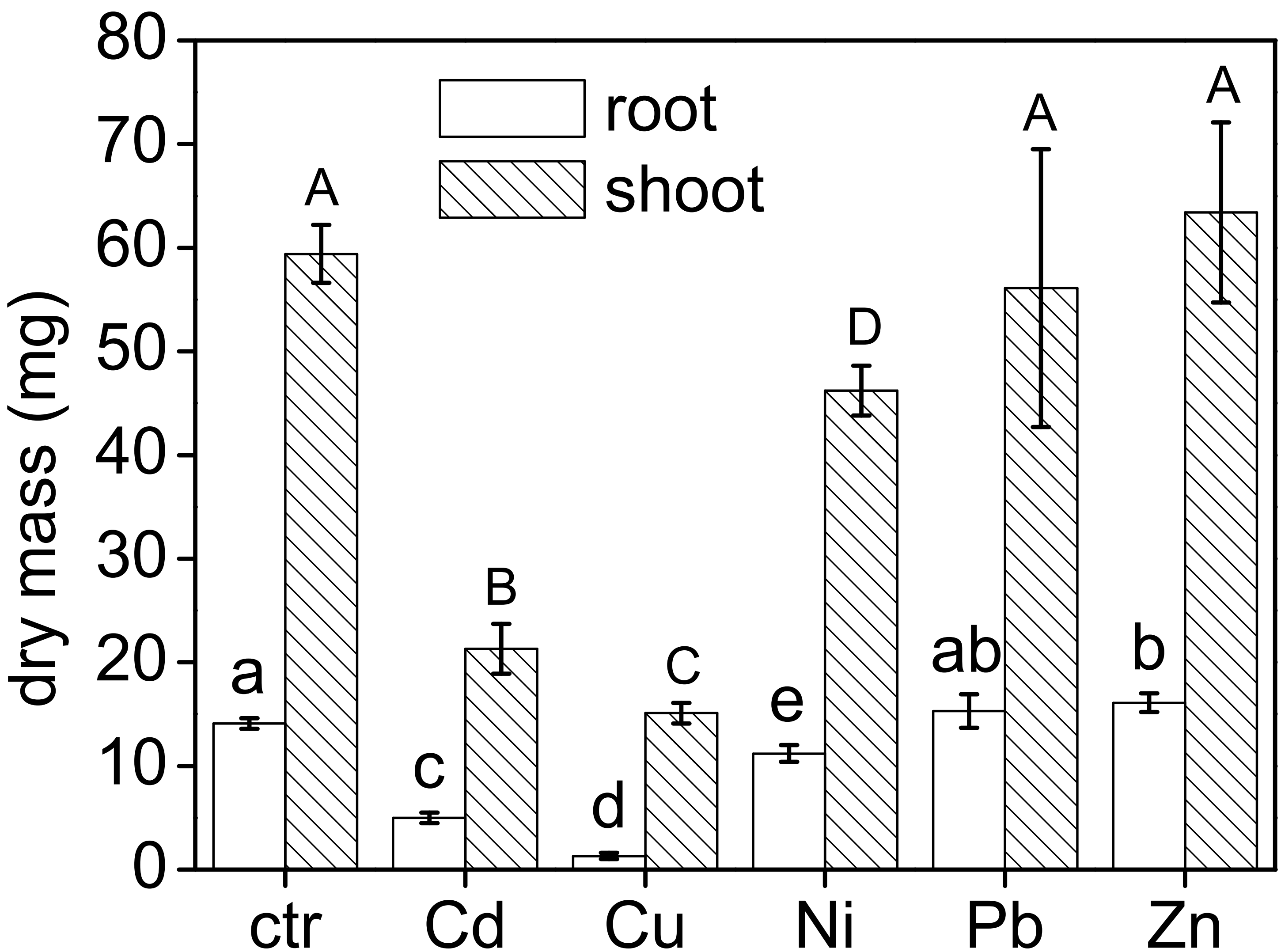
treatment	Day 0	Day 4
ctr	4,70	7,66±0,05 a
Cd	4,67	6,73±0,01 b
Cu	4,70	6,11±0,08 c
Ni	4,60	7,42±0,12 d
Pb	4,76	7,297±0,20 d
Zn	4,78	7,72±0,05 a

Table 2

Table 2. Translocation index [TI = shoot total Me_i content (g)/root total Me_i content (g)] and phytoextraction capacity [PC = shoot total Me_i content (g) *100/ Me_i supplied to the nutrient solution during the whole growth period (g)] of Me_i and Fe in Szarvasi-1 energy grass grown in nutrient solutions amended with different metals (Me_i) in 0 (ctr) and 10 μM concentration. (The concentration of Fe was also 10 μM in all treatments and the untreated control.)

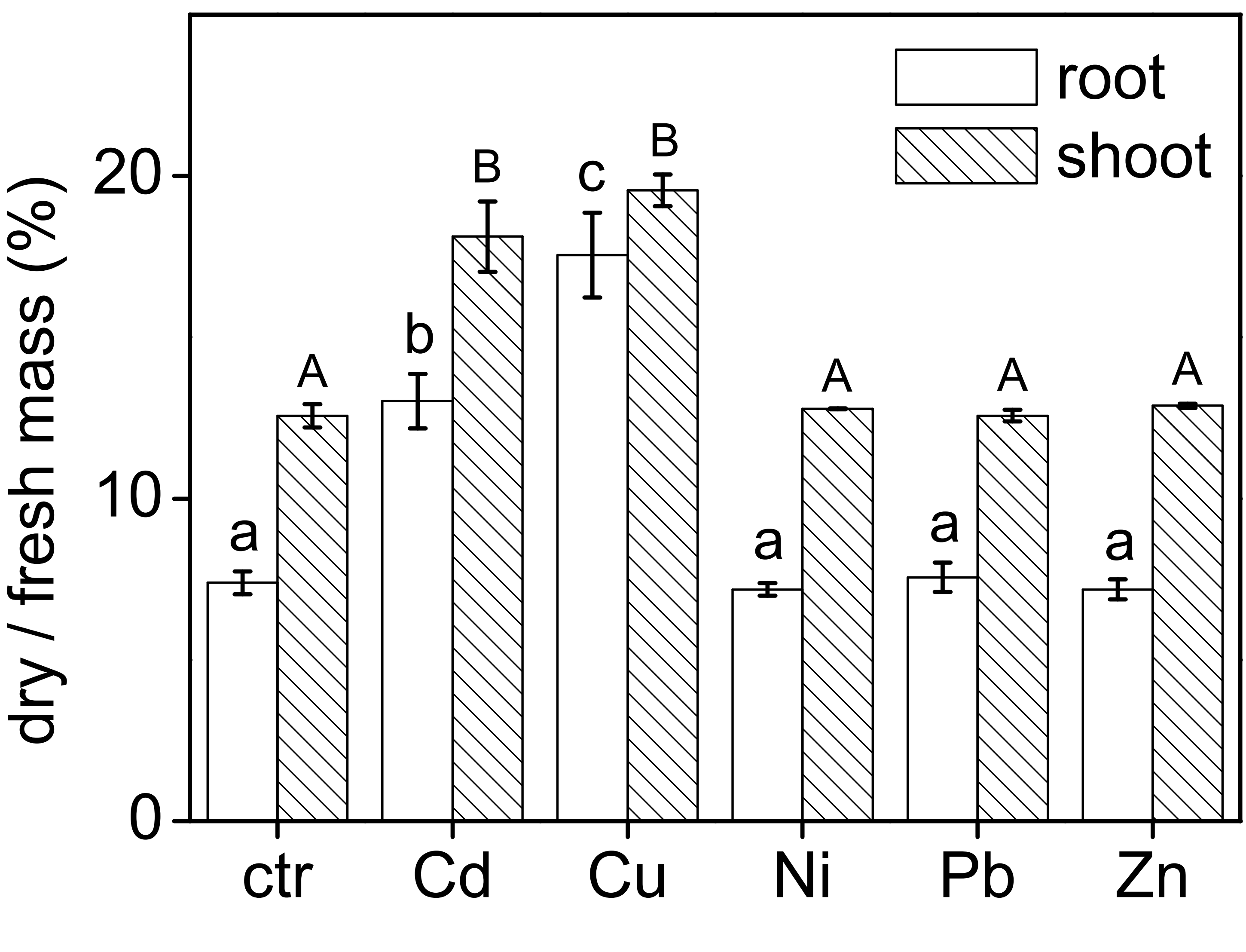
treatment (Me_i)	TI (Me_i)	TI (Fe)	PC (Me_i)	PC (Fe)
Cd	0.259	0.135	0.071	0.691
Cu	0.157	0.107	0.041	0.350
Ni	0.077	0.419	0.038	1.286
Pb	0.004	1.027	0.024	1.725
Zn	0.280	0.744	0.467	2.262
Fe (ctr)	-	1.118	-	1.585

Figure 1



80
70
60
50
40
30
20
10
0
dry mass (mg)
root
shoot
A
B
C
D
A
A
a
c
d
e
ab
b
ctr
Cd
Cu
Ni
Pb
Zn

Figure 2



root
shoot
dry / fresh mass (%)
20
10
0
a
A
b
B
c
B
a
A
a
A
a
A
ctr
Cd
Cu
Ni
Pb
Zn

Figure 3

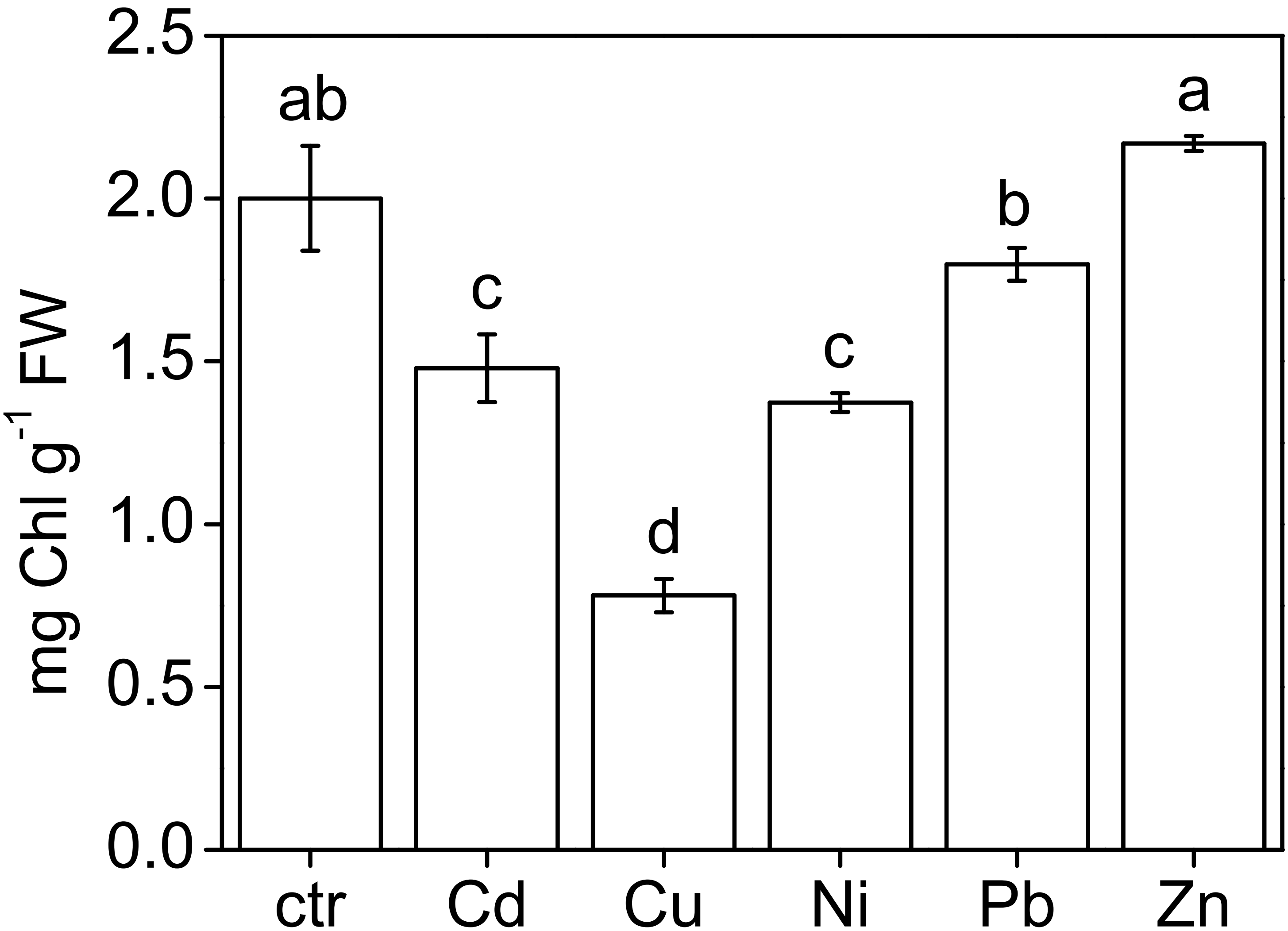


Figure 4

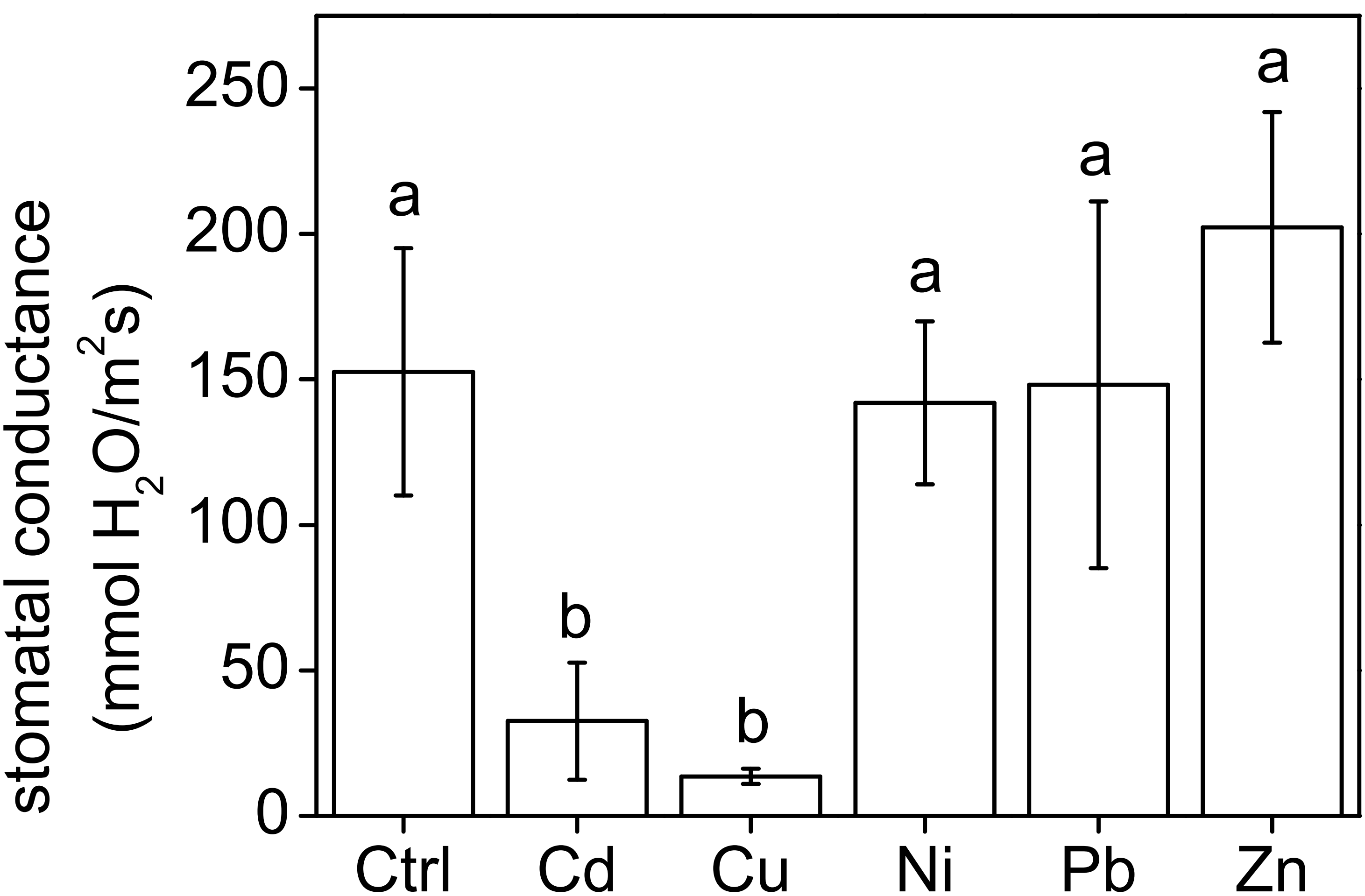
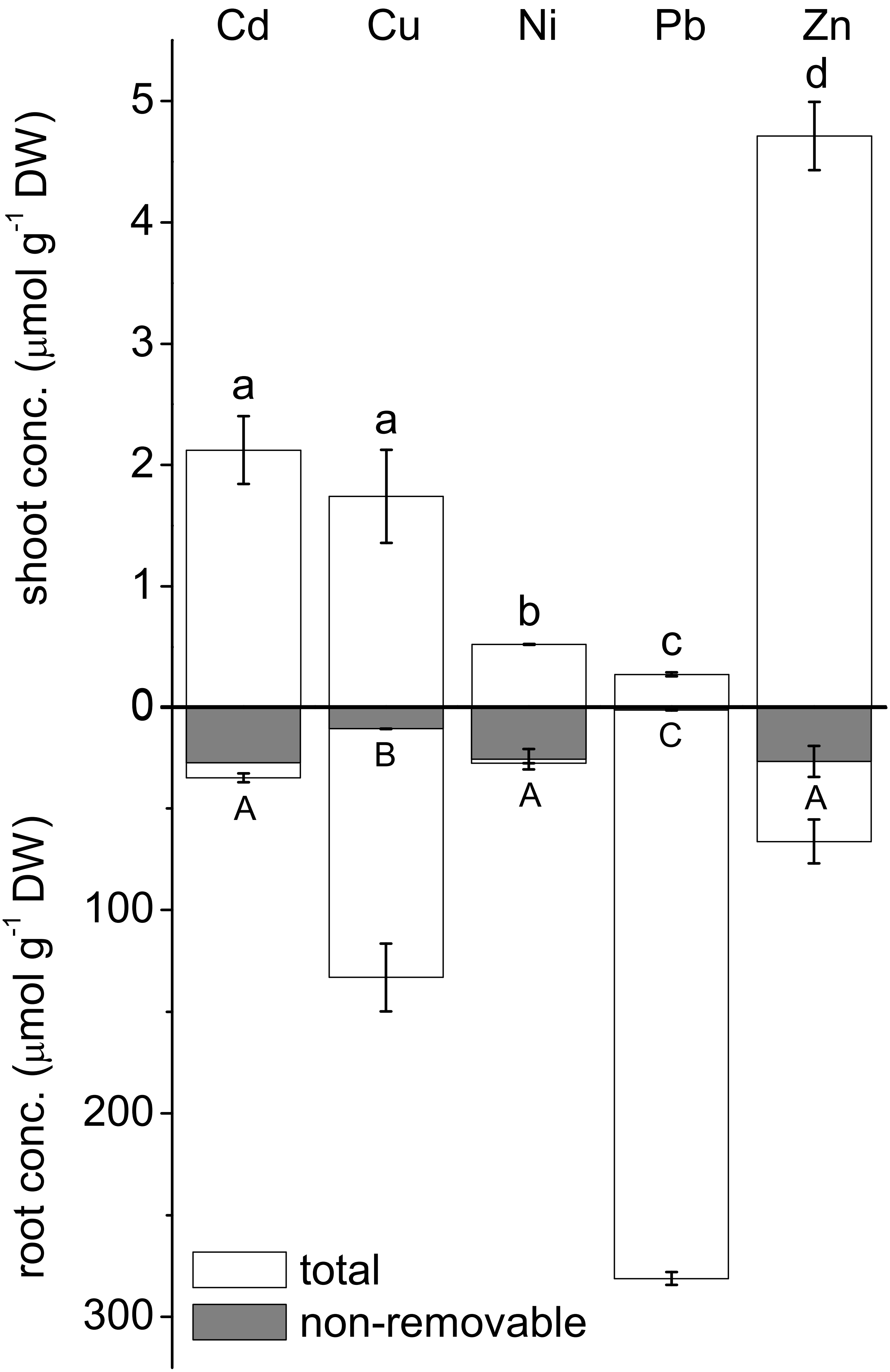
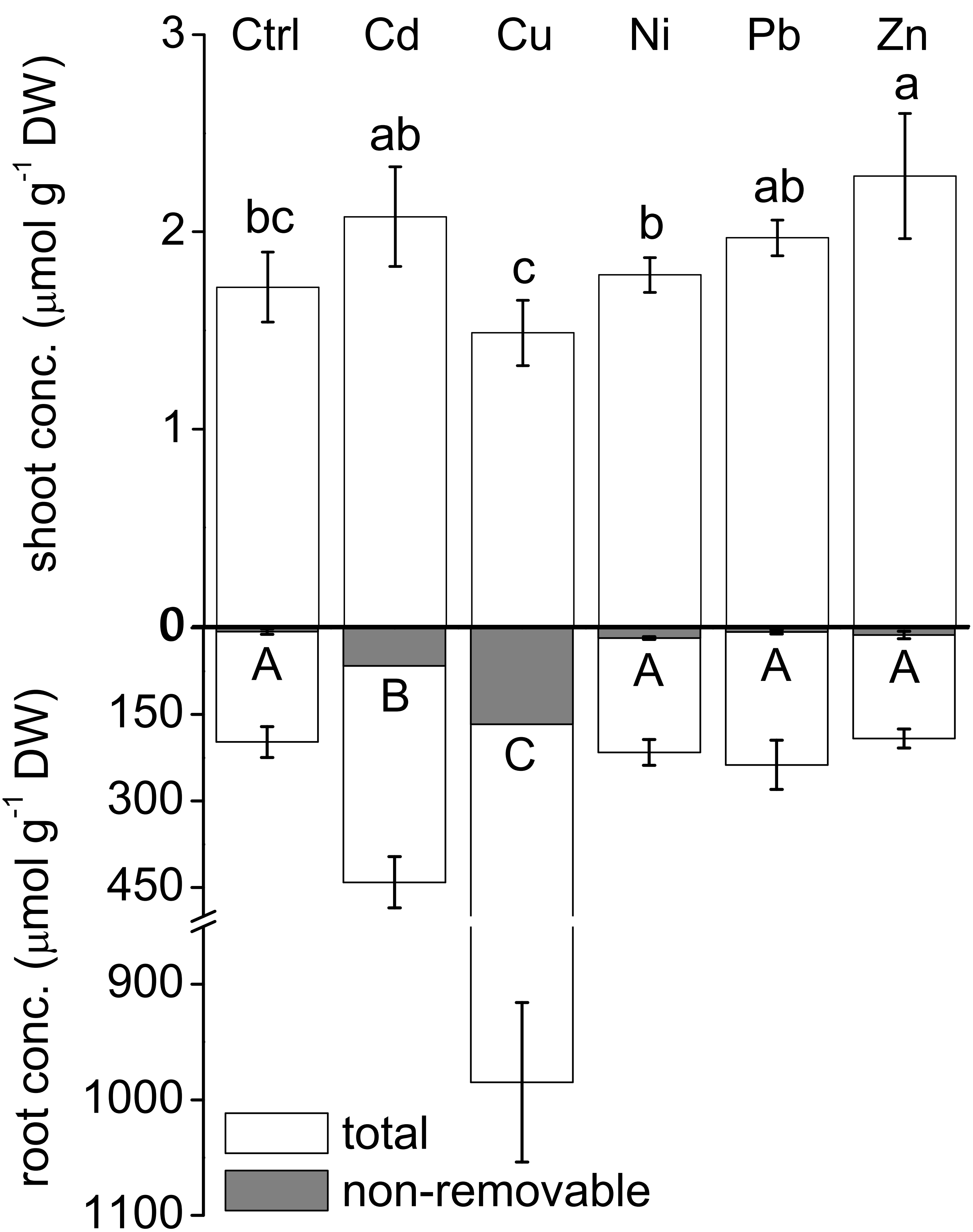

stomatal conductance
(mmol H2O/m2s)
250
200
150
100
50
0
a
b
b
a
a
a
Ctrl
Cd
Cu
Ni
Pb
Zn

Figure 5



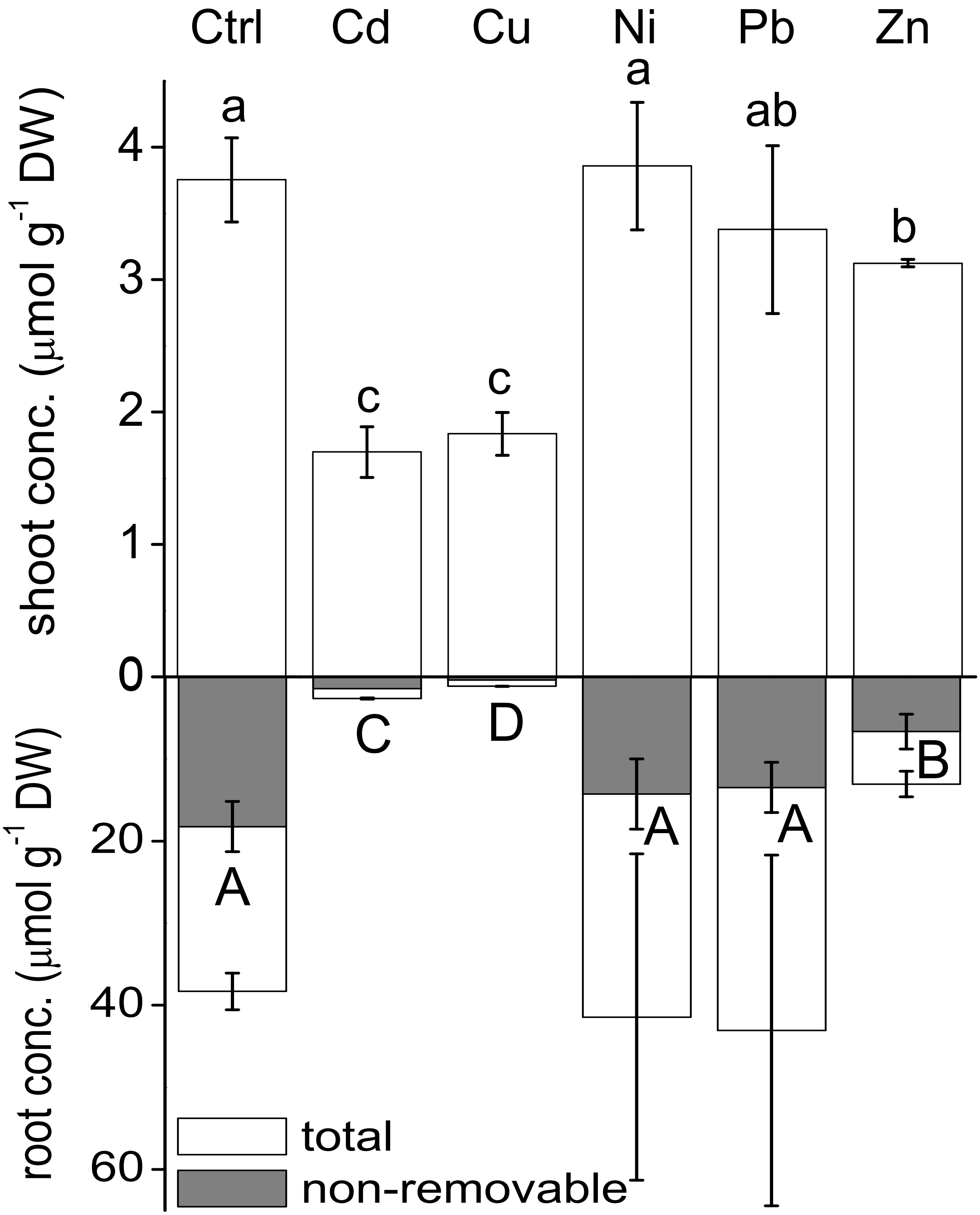
Cd
Cu
Ni
Pb
Zn
shoot conc. (μmol g-1 DW)
root conc. (μmol g-1 DW)
5
4
3
2
1
0
100
200
300
a
a
b
c
d
A
B
A
C
A
total
non-removable

Figure 6



Ctrl
Cd
Cu
Ni
Pb
Zn
shoot conc. (µmol g-1 DW)
root conc. (µmol g-1 DW)
3
2
1
0
150
300
450
900
1000
1100
bc
ab
c
b
ab
a
A
B
C
A
A
A
total
non-removable

Figure 7



Ctrl
Cd
Cu
Ni
Pb
Zn
shoot conc. (μmol g-1 DW)
root conc. (μmol g-1 DW)
a
c
c
a
ab
b
A
C
D
A
A
B
4
3
2
1
0
20
40
60
total
non-removable

Figure 8

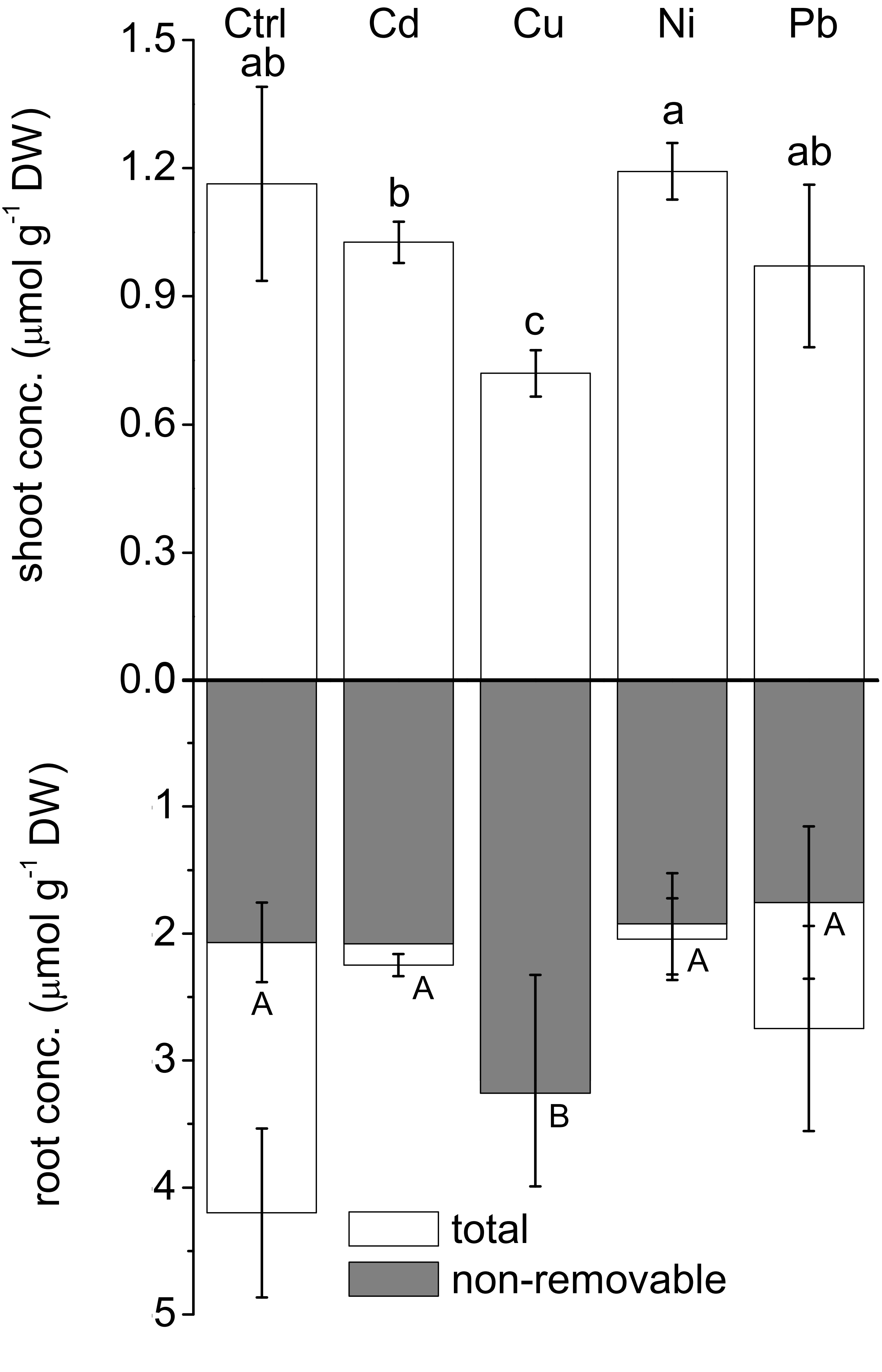
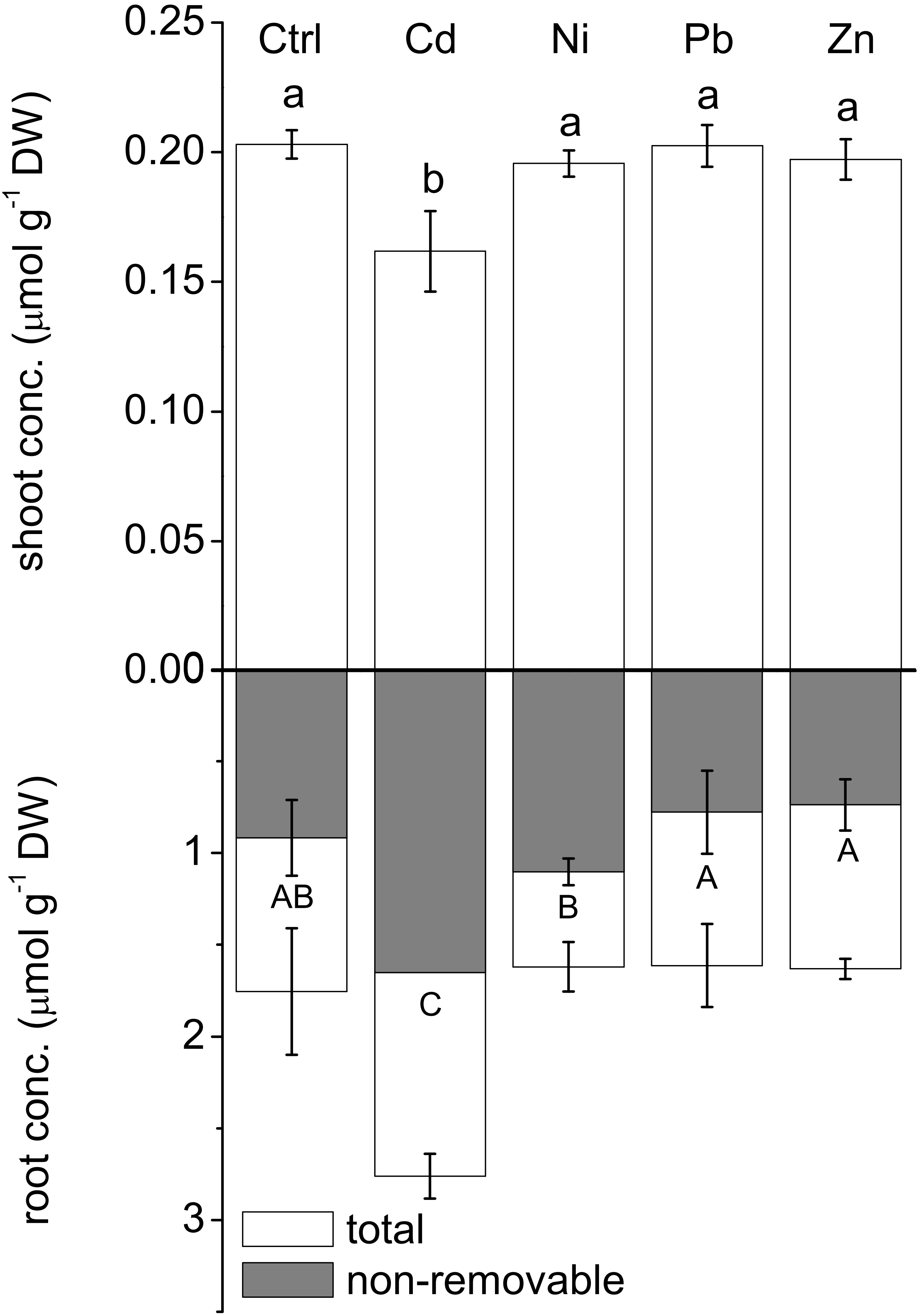

Ctrl
Cd
Cu
Ni
Pb
ab
b
c
a
ab
A
A
B
A
A
1.5
1.2
0.9
0.6
0.3
0.0
1
2
3
4
5
shoot conc. (μmol g-1 DW)
root conc. (μmol g-1 DW)
total
non-removable

Figure 9



0.25
0.20
0.15
0.10
0.05
0.00
1
2
3
shoot conc. (μmol g-1 DW)
root conc. (μmol g-1 DW)
Ctrl
Cd
Ni
Pb
Zn
a
b
a
a
a
AB
C
B
A
A
total
non-removable