



Polyaspartate, a biodegradable chelant that improves the phytoremediation potential of poplar in a highly metal-contaminated agricultural soil

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ABSTRACT

Phytoremediation is a cost-effective and environment friendly *in situ* technique for the reclamation of heavy metal-polluted soils. The efficacy of this technique, which relies on tolerant plant species, can be improved by the use of chelating agents. A pot experiment was carried out to evaluate the phytoextraction and phytostabilisation capacities of a white poplar (*Populus alba* L.) clone named AL35 previously selected for its marked tolerance to copper (Cu) and zinc (Zn). Cuttings were grown on agricultural soil highly contaminated with Cu and Zn, in the presence or not (controls) of a chelant mixture (EDTA/EDDS) known to enhance metal bioavailability and, hence, uptake by plant roots, or the not yet investigated synthetic, highly biodegradable polyaspartic acid (PASP). Both chelant treatments improved the phytostabilisation of Cu and Zn in AL35 plants, whilst the phytoextraction capacity was enhanced only in the case of Cu. Considering that the effectiveness of PASP as phytostabilizer was comparable or better than that of EDTA/EDDS, the low cost of its large-scale chemical synthesis and its biodegradability makes it a good candidate for chelant-enhanced metal phytoextraction from soil while avoiding the toxic side-effects previously described for both EDTA and EDDS.

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1. Introduction

Pollution caused by heavy metals (HMs) is one of the major environmental concerns that affect both industrialized and emerging countries. Although a limited amount of HMs in soils derives from natural processes, the major part originates from human activities (Adriano, 2001; Clemens, 2006). Along food chains, HM accumulation can lead to the bio-magnification phenomenon (Zerbi and Marchiol, 2004), which negatively influences community dynamics.

Most polluted sites are multi-contaminated, and the presence of a single contaminant is extremely rare. Thus, different integrated techniques, called “treatment trains”, must be employed to reclaim the polluted areas (Roote, 2003). Amongst these, phytoremediation

is particularly effective in the decontamination of HM-polluted soils. Phytoremediation comprises a set of technologies (phytoextraction, -stabilisation, -volatilisation, or -degradation, and rhizodegradation and -filtration) that exploit plants and root-associated microorganisms in order to take up, sequester, remove or degrade contaminants, including organic compounds and HMs. This emerging technology provides an inexpensive, environment friendly and publicly sustainable treatment, useful for many multi-contaminated sites (Wu et al., 2010).

A successful phytoextraction process is strictly related to adequate biomass yields and high HM contents in the harvestable parts of the plants (Komarek et al., 2007). Poplars are characterized by a great genetic biodiversity (Castiglione et al., 2010), a high biomass production and a striking aptitude for phytoextraction and tolerance to HMs (Dickmann, 2001; Giacchetti and Sebastiani, 2006; Utmazian et al., 2007; Castiglione et al., 2009).

The main drawback of phytoextraction is the low mobility, and thus bioavailability, of micronutrients and trace elements in the

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polluted soils (Adriano, 2001). The bioavailability of metals mainly depends upon soil pH, cation exchange capacity (CEC) and organic matter content, the release of chelating agents from roots, and the presence of rhizobacteria or mycorrhiza (Hong-Bo et al., 2010). An increase of HM mobility can be achieved by the addition of synthetic chelating agents capable of solubilising and complexing them into the soil water solution, thus promoting their uptake into roots, and translocation from roots to shoots (Blaylock et al., 1997; Huang et al., 1997). In recent years, chelant-enhanced phytoextraction of HMs from contaminated soils has received much attention, as an alternative to costly conventional methods, for soil reclamation (Nowack et al., 2006; Evangelou et al., 2007).

A number of more or less biodegradable chelators, such as ethylenediaminetetraacetic acid (EDTA), trans-1,2-diaminocyclohexane-N,N',N'-tetraacetic acid (CDTA), ethyleneglycol-bis(b-aminoethyl ether),N,N,N',N'-tetraacetic acid (EGTA), ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid) (EDDHA), nitrilotriacetic acid (NTA), and organic acids (e.g., citric acid, malic acid) have been used to desorb metals from soil colloids in order to facilitate their removal (Chen et al., 2003; Meers et al., 2004; Luo et al., 2005; Evangelou et al., 2007; Trezena de Araujo and Araujo do Nascimento, 2010). However, because of their high solubility and persistence in the soil, some chelants can cause HM leaching, thereby posing an environmental risk for groundwater quality (Romkens et al., 2002). The chelating agent EDTA, for instance, is one of the most frequently used chemical compounds to make contaminants, such as lead, more bioavailable in different kinds of soils (Saifullah et al., 2009). However, due to its high persistence in the soil, and toxicity to plants (Epelde et al., 2008), several alternatives have been proposed, e.g., imino-disuccinic acid [IDS (Helena et al., 2003; Komarek et al., 2007)]. In particular, a highly biodegradable structural isomer of EDTA, EDDS ([S, S]-ethylene-diamine-disuccinate), showing a lower leaching risk, has been introduced as a promising mobilizing agent, especially for Cu and Zn (Luo et al., 2005, 2006a). EDDS causes less stress to soil microorganisms and plants because of its low toxicity and high biodegradability (Kos and Lestan, 2003), while enhancing phytoextraction capacity relative to EDTA (Luo et al., 2007). At equimolar ratios of chelating agent to metals, EDDS was found to be more effective than EDTA in solubilising Cu and Zn from soils at pH 7.0 (Hauser et al., 2005). The simultaneous application of EDTA and EDDS generally shows a superior response (Luo et al., 2006b), and combined applications of the two chelants in different ratios can be used in phytoextraction in order to reduce the undesired side-effects of EDTA alone (Mohavedi et al., 2011). However, although these chelating agents offer a good alternative to the less biodegradable and more toxic EDTA, the main problem for their large-scale use remains the high costs. Therefore, searching for environment friendly and economically viable alternatives is imperative, so that phytoremediation can become a valuable and effective technology for reclamation of HM-contaminated areas while, at the same time, restoring the soil ecosystem.

Polyaspartic acid (PASP) and other poly-amino acids have a variety of industrial (water treatment, paper processing, paint additives) as well as potential biomedical (as a component in dialysis membranes, artificial skin, drug delivery systems, and orthopaedic implants) applications (Roweton et al., 1997). On the other hand, poly-amino acids are biodegradable chelators whose potential utility in phytoextraction experiments has yet to be tested. PASP is certainly one of the most promising of this class of compounds (Nita et al., 2006). It possesses several carboxylic groups able to coordinate various HMs, is not toxic, is rapidly biodegraded, and can form complexes more or less efficiently with different HMs (Roqu   et al., 2007), making it an attractive candidate for phytoremediation.

The aim of this work was to compare, in a pot experiment, the effect of an EDTA/EDDS mixture with that of PASP on Cu and Zn

mobility in a highly contaminated soil, and on the phytoremediation capacity of a white poplar clone (AL35), previously selected for its good performance during a field trial on a multi-metal contaminated site. In the attempt to reproduce, as closely as possible, the conditions that would occur in an open field trial, the soil used came from the same polluted site on which the clone was selected.

2. Materials and methods

2.1. Synthesis of PASP

All reagents used for the polycondensation of L-aspartic acid were purchased from Sigma Aldrich (Milano – Italy). In a nitrogen atmosphere, 100 g (0.75 mol) of L-aspartic acid was stirred for 2 h in 800 mL of 7:3 (w/w) diethylbenzene/sulfolane solution. Five mL of phosphoric acid (85%) were added dropwise, and the mixture refluxed for 7 h. The water formed in the reaction mixture was removed using a Dean–Stark trap with a reflux condenser. The solvent was removed, the precipitate washed with methanol (800 mL), and then with water (800 mL) several times until it reached a neutral pH. The residue was washed with ethanol (800 mL) and then dried at 85 °C under reduced pressure. The yield of polysuccinimide was about 80%. To a 50 g aliquot of polysuccinimide, 500 mL of a 0.2 M NaOH water solution were added portion-wise with stirring in an ice-bath. After stirring for 5 h at room temperature, the reaction mixture was poured in methanol over a 1-h period, and the entire mixture was stirred. The precipitate was filtered, washed with methanol, and then dried under vacuum at 50 °C for 24 h. The yield was approximately 90%.

2.2. Plant material and experimental design

Cuttings of a metal tolerant *Populus alba* L. clone (AL35; Castiglione et al., 2009) were collected in February 2008 and allowed to root in sand before transplanting them to 25-L pots filled with soil collected, to a depth of 20 cm, from an agricultural metal-polluted site, located next to an industrial plant for metallic alloy production (Serravalle Scrivia, AL, Italy). The soil pH and organic matter content were reported in a previous paper (Castiglione et al., 2009). In March, rooted cuttings were planted in pots (five per treatment) containing: (i) no chelants (controls), (ii) a mixture of EDTA (3 mmol kg⁻¹ DW polluted soil) and EDDS (2 mmol kg⁻¹ DW polluted soil), or (iii) PASP (5 mmol kg⁻¹ DW polluted soil). The EDTA/EDDS mixture was used at that concentration because it was previously reported to be the most effective in phytoextracting HMs from contaminated soils (Luo et al., 2006b). The chelants, dissolved in water, were added to the pots in July by watering the plants twice (each time with half the final amount of chelants) with an interval of one week. Plants were grown in a greenhouse under conditions of natural light. They were watered regularly, and fertilized once with 16 g per pot of slow-release granular “Nitrophoska giardino” (Compo, Cesano Maderno, MB, Italy), containing N:P:K (15:9:15 kg per 100 kg of total product, respectively), Mg (2 kg per 100 kg) and SO₃ (20 kg per 100 kg).

Three-four leaves were collected from each plant four days (first sampling, July 24), one month (second sampling, August 13) and two months (third sampling, September 25) after chelant addition for the determination of Cu and Zn concentrations. Roots were collected at the end of November, after leaf fall. Stems were not analysed for Cu and Zn concentrations because they can be considered as a simple translocation organ in poplar plants in which a very limited amount of HMs are stored (Castiglione et al., 2009). Stem diameter at ground level was measured with a

calliper at the end of the experiment to estimate the growth performance of the plants.

2.3. Metal determinations in plants and soil

Total and available, i.e., diethylenetriamine-pentaacetic acid-(DTPA-) extractable, concentrations of Cu and Zn were determined in the soil and leaves before starting the experiment (zero time), and four days, one month and two months after chelant addition, while root metal concentrations were estimated at the end of the trial.

Soil was collected from each pot of every treatment separately and pooled for total and bioavailable metal analysis. For total metal analyses, the soil granulometric fraction was oven-dried at 75 °C up to constant weight, and pulverised in a planetary ball mill (PM4, Retsch, Germany) with agate mortars. Plant material was also oven-dried at 75 °C; then, leaves were manually pulverised in liquid nitrogen, while roots were incinerated in an oven at 550 °C (Controller B 170, Nabertherm, Germany). All soil and plant samples were digested with an acid mixture (65% HNO₃: 50% HF, 2:1, v:v) in a microwave oven (Ethos, Milestone, Shelton, CT, USA). The available concentration of metals was extracted from the granulometric fraction according to the Lindsay and Norvell (1978) method. Metal concentrations were determined by an atomic absorption spectrophotometer (AAnalyst 100, PerkinElmer, Wellesley, MA, USA), via an air-acetylene flame (Zn), or a graphite atomiser (Cu). Three replicates of each analysis were carried out. To test the accuracy of the data, standard reference materials (NCS DC73321 soil – China National Analysis Center for Iron and Steel 2008 and 1575a Pine Needles – NIST 2004) were also analysed.

2.4. Statistical analyses

Statistically significant differences in stem diameter at ground level were evaluated using ANOVA followed by a *post-hoc* Tukey HSD test ($n = 5$; $P < 0.05$). For both leaf and soil samples, differences in metal concentrations were assessed by two-way ANOVA on the normalised data set, with treatment and sampling as fixed factors. Moreover, to evaluate differences in metal concentrations between roots and leaves, on the data of the last sampling (September), a two-way ANOVA was carried out on the normalised data set considering treatment and plant organ as fixed factors. Both ANOVA tests were followed by the *post-hoc* test of Holm-Sidak ($\alpha = 0.05$). Statistical analyses were performed using the SigmaPlot 11.0 software package (Jandel Scientific, San Rafael, CA, USA).

2.5. Translocation and bio-accumulation factors

Trace metal translocation in plants from roots to shoot was calculated using the Translocation Factor (TF), as illustrated below:

$$TF = C_L / C_R$$

where C_L is the metal concentration in the leaves and C_R is the metal concentration in the root.

The trace metal Bio-Accumulation Factor (BAF) was determined by calculating the ratio of metal concentration in the different parts of the plant with that of the soil, as illustrated below:

$$BAF = C_p / C_s$$

where C_p is the metal concentration in the different plant organs and C_s is the metal concentration in the soil at zero time (An, 2004).

3. Results

3.1. Uptake and metal translocation in the plant

The concentrations of Cu and Zn in leaves of *P. alba* clone AL35 grown on HM-polluted soil, with or without chelants, four days, one month and two months (July, August and September) after chelant addition, and in roots collected at the end of the experiment (November) are shown in Fig. 1. No differences in stem diameter between control and chelant-treated plants were observed (Table 1).

3.1.1. Copper

Leaves showed statistically significant differences in Cu concentration in relation to time ($F = 114.53$; $P < 0.001$) and treatments ($F = 151.00$; $P < 0.001$). The highest average Cu concentrations were reached at the second sampling time in the roots, while the lowest ones were observed at the first sampling time. In general, the leaves

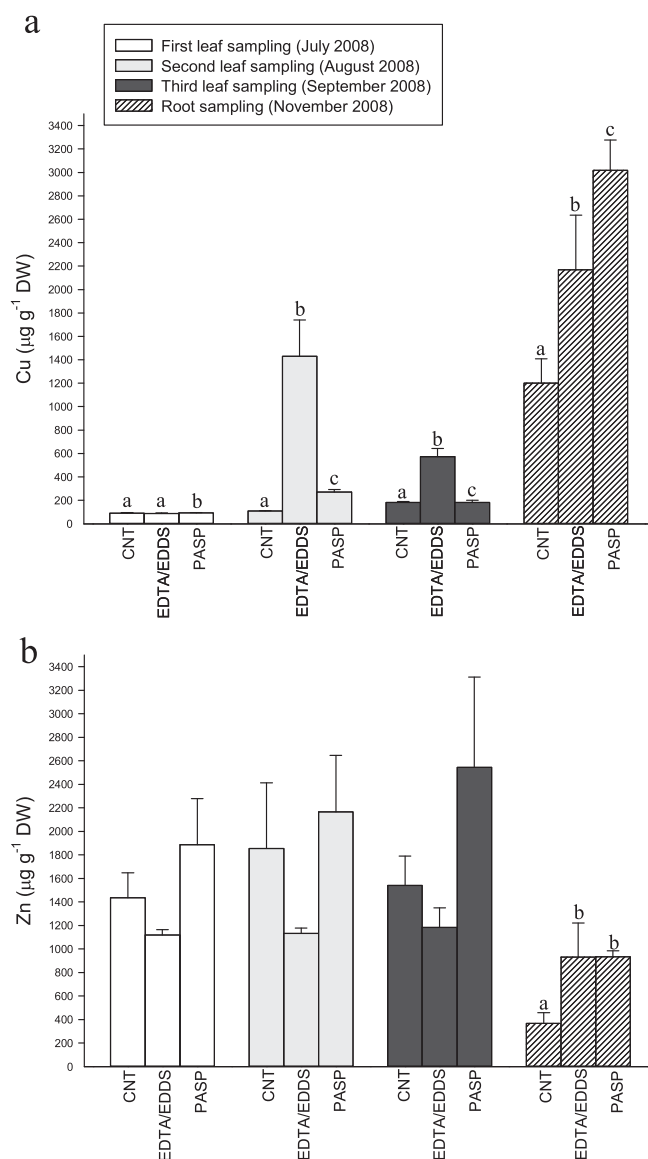


Fig. 1. Copper (a) and zinc (b) concentrations (mean values $\pm$ standard errors) in leaves and roots of white poplar clone AL35 at three different sampling times for leaves (July, August and September 2008), and at the end of the pot trial for roots (November 2008). Different letters indicate significant differences ($\alpha = 0.05$) within each sampling time.

Table 1
Stem diameter of AL 35.

Treatment	Stem diameter (mm)
CNT	11.6 ± 1.7 ^a
EDTA/EDDS	9.7 ± 1.2 ^a
PASP	11.0 ± 1.0 ^a

Diameter at ground level of clone AL35 grown on a metal-contaminated soil in the presence or absence (controls) of chelants was measured at the end of the vegetative season. Different letters indicate statistically significant differences according to ANOVA followed by a post-hoc Tukey HSD test ($n = 5$; $p < 0.05$).

of control plants showed the lowest Cu concentrations, while the highest were found in the leaves of EDTA/EDDS treatments ($\alpha = 0.05$); PASP-treated plants showed, on average, an intermediate level of leaf Cu concentrations (Fig. 1a). Four days after chelant addition, the highest concentration was observed in the leaves of PASP-treated plants ($91.91 \mu\text{g g}^{-1}$ DW) while, at later sampling times, the highest concentration was observed in the EDTA/EDDS treatment (1428.86 and $571.34 \mu\text{g g}^{-1}$ DW) at second and third sampling, respectively. Copper concentration in roots and leaves, at the last sampling time, showed significant differences ($F = 598.15$ and $P < 0.001$), with the higher concentration in roots. Roots also showed significant differences ($\alpha = 0.05$) between treatments. The highest concentration was observed in the PASP treatment ($3018.68 \mu\text{g g}^{-1}$ DW), followed by EDTA/EDDS ($2167.41 \mu\text{g g}^{-1}$ DW), and control plants ($1200.36 \mu\text{g g}^{-1}$ DW).

At the second and third sampling, EDTA/EDDS increased the Cu BAF value for leaves compared to both controls and PASP treatments (Table 2). Moreover, the chelant mixture favoured the translocation of Cu to the aerial parts of the plants as indicated by the higher TF values relative to both controls and PASP (Table 3). By contrast, although PASP did not increase root-to-shoot translocation (TF) of Cu (Table 3), it resulted in a higher root BAF than in controls (Table 2). Thus, while the BAF of control roots was 7–9 fold higher than that of the leaves, with PASP it was 13–17 fold higher, and with EDTA/EDDS only ca. 2–4 fold higher.

3.1.2. Zinc

Zn concentrations were much higher than those of Cu in control leaves, reaching about $1500 \mu\text{g g}^{-1}$ DW already at first sampling, and without significant differences between sampling times (Fig. 1b). Considering all sampling times, significant differences ($F = 6.150$; $P < 0.01$) were observed among treatments with the highest concentrations obtained with PASP ($2542.83 \mu\text{g g}^{-1}$ DW), and the lowest with EDTA/EDDS ($1117.46 \mu\text{g g}^{-1}$ DW). Considering the chelant treatments and controls together, compared with the leaves the lowest concentrations of Zn were observed in the roots ($F = 28.601$; $P < 0.001$; $600 \mu\text{g g}^{-1}$ DW for controls vs a mean value of $2000 \mu\text{g g}^{-1}$ DW for the two treatments). The highest root concentrations ($\alpha = 0.05$) were reached in the presence of chelants (ca. $900 \mu\text{g g}^{-1}$ DW; Fig. 1b). Consequently, TF values decreased relative to controls (Table 3). Finally, while in controls the Zn BAF was much higher (ca. five fold) in the leaves than in the roots, leaf BAF after the EDTA/EDDS treatment was slightly higher or equivalent to that of

Table 2
Bioaccumulation factor (BAF).

	Cu			Zn		
	Leaf (II)	Leaf (III)	Root	Leaf (II)	Leaf (III)	Root
CNT	0.13	0.17	1.14	2.79	2.05	0.49
EDTA/EDDS	1.27	0.74	2.79	1.39	1.86	1.47
PASP	0.19	0.15	2.50	2.39	2.91	1.07

BAF for Cu and Zn in leaves (at second and third sampling) and roots (end of experiment) of clone AL35 grown on a metal-contaminated soil in the presence or absence (controls) of chelants. (II), second sampling; (III), third sampling.

Table 3
Translocation Factor (TF).

	Third sampling	
	Cu	Zn
CNT	0.15	4.21
EDTA/EDDS	0.26	1.27
PASP	0.06	2.72

TF for Cu and Zn at third sampling times in clone AL35 grown on a metal-contaminated soil in the presence or absence (controls) of chelants.

the roots; on the contrary, leaf BAF was 2.3- and 2.9-fold higher than that of the roots in the case of PASP treatment at the second and third sampling times, respectively (Table 2).

3.2. Soil Cu and Zn concentrations

Total and DTPA-extractable concentrations of Cu and Zn in pot soils before (zero time) and during the experiment (second and third sampling times) are shown in Fig. 2a. At the start of the experiment, total soil Cu concentration was approximately $3300 \mu\text{g g}^{-1}$ DW, and the bioavailable fraction approximately one tenth of that (Fig. 2a). One month after chelant addition, total Cu concentration declined dramatically (ca. to one-third) in both control and chelant-supplemented soil, and remained constant at the subsequent sampling times. Differences along sampling times were on average significant ($F = 108.893$; $P < 0.001$).

Total Cu concentration was different among treatments ($F = 4.980$; $P < 0.05$), with a slightly higher concentration in the case of PASP. DTPA-extractable Cu was extremely high in the soil of control plants, and chelant addition did not cause any significant variation among treatments (Fig. 2a), but a slight decrease was observed with time ($F = 6.036$; $P < 0.01$).

Although a slight decrease in Zn concentrations was observed in the course of the experiment for both total and DTPA-extractable fractions ($F = 6.456$, $P < 0.01$ and $F = 6.135$, $P < 0.01$, respectively), concentrations were comparable in all assayed soils (Fig. 2b).

4. Discussion

The results of the present study indicate that PASP, whose synthesis is a relatively low-cost operation (see for information: <http://www.nanochemsolutions.com/>), is able to increase the amount of Cu and Zn that is taken up and accumulated in poplar plants growing on a Cu- and Zn-contaminated soil, thus contributing to the phytoextraction, in the case of Cu, and phytostabilisation of these metals.

4.1. Chelant-enhanced metal uptake and translocation in different plant organs

The high capacity for HM uptake of the AL35 white poplar clone, previously demonstrated during an ongoing field trial (Castiglione et al., 2009), was confirmed by this pot experiment. In fact, comparing the HM concentrations of roots and leaves of AL35 in the two experiments, both performed on the same multi-metal polluted soil, and considering solely the metal accumulation data of control plants in the present study, only root Zn concentrations were three-fold higher in the field-grown plants than in those of the pot experiment, while all other values were comparable. This difference might be explained by the presence of different microbial (bacteria and mycorrhiza) communities in the rhizosphere of field-grown plants as compared with those transferred to the pots, a factor which can differentially affect plant growth and metal

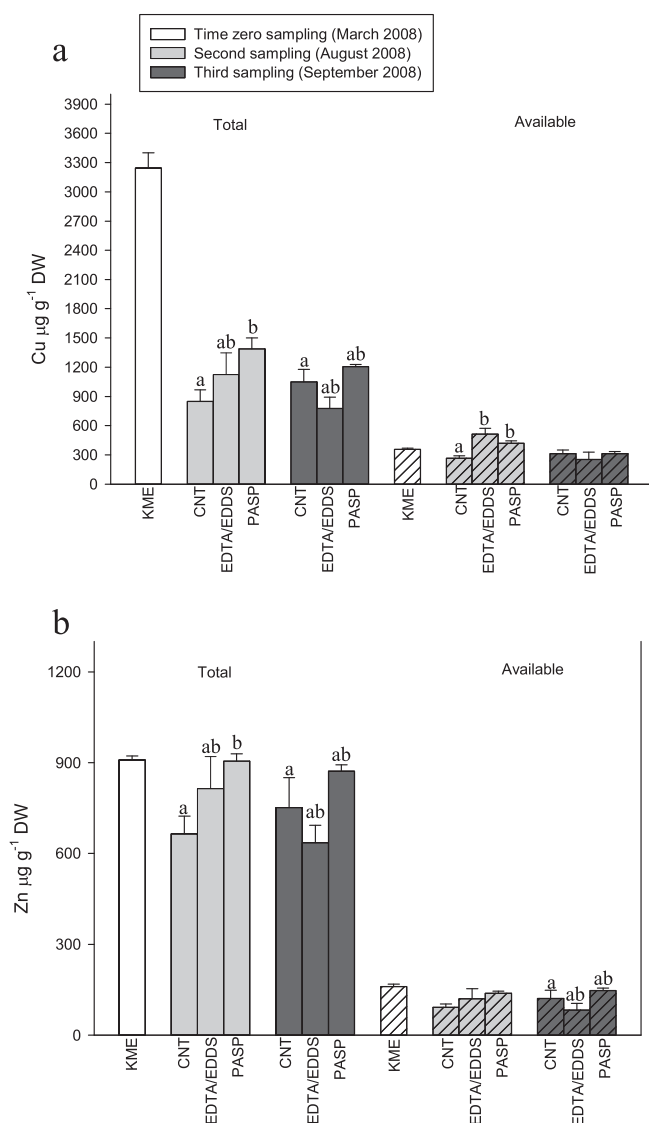


Fig. 2. Concentrations (mean values $\pm$ standard errors) of total and bioavailable copper (a) and zinc (b) at the start of the experiment (March 2008) and at the second and third sampling times (August and September 2008). Different letters indicate significant differences ($\alpha = 0.05$) within each sampling time.

accumulation (van der Lelie et al., 2009; Cicatelli et al., 2010; Gamalero et al., 2012).

In the present pot trial, the application of EDTA/EDDS and, to a lesser extent, PASP to the contaminated soil led to an increase of leaf Cu concentrations. Our data confirm, therefore, that the EDTA/EDDS chelant mixture is extremely useful for Cu phytoextraction, as previously observed by Luo et al. (2006b) in the case of maize. Indeed, both EDTA/EDDS and PASP rapidly and dramatically increased Cu availability in polluted soil, and, therefore, the element was quickly taken up and easily translocated, probably as Cu/chelant complexes.

Although Cu is a micronutrient, it is scarcely translocated from roots to shoots (Kopponen et al., 2001; Todeschini et al., 2007), and any excess is blocked by the cells of the root endoderm provided with the Casparian strip (van der Lelie et al., 2009). The selective activity of the endoderm cell membranes reduces the risk of phytotoxicity caused by high concentrations of this redox active element, that, even at low concentrations (less than 30 mg kg^{-1} DW), can severely damage plant metabolism (Luo et al., 2006b; and references therein). In the present study, no visual

toxicity symptoms were observed in any of the plants, and plant growth (in terms of stem diameter) was not affected, confirming the high tolerance of AL35 to Cu, and its potential for phytoextraction of this HM from soil (Castiglione et al., 2009). The superior growth performance and phytoremediation capacity of clone AL35, compared to those of other poplar clones/species cultivated on multi-metal contaminated soils, even in the presence of phytotoxic chelants such as EDTA or EDDS (Komarek et al., 2008), were further confirmed by our study. The same AL35 trees, in fact, four years after chelant treatment and repeated coppicing were still alive and healthy on the same Cu- and Zn-polluted soil (unpublished data). These observations contradict to some extent those reported by Komarek et al. (2008), showing a noticeable (90%) reduction at the second growth year in the case of a hybrid poplar clone cultivated on high or medium lead-contaminated soils amended with comparable amounts of chelant (3.0 or $6.0 \text{ mmol EDTA kg}^{-1}$ DW soil). Moreover, the AL35 clone showed, around 160 days after cutting plantation, Cu concentrations in leaves that were 20- (EDTA/EDDS mixture) and 5-fold (PASP) higher than those observed by Komarek et al. (2010), who employed a different poplar clone cultivated on an agricultural Cu-contaminated soil in the presence of EDDS (6.0 mmol kg^{-1} DW soil).

The addition of either of the two chelants (EDTA/EDDS or PASP) to AL35 cuttings grown on polluted soil not only improved Cu translocation to the above-ground parts of the plant, but also the phytostabilisation of the metal, insofar as Cu concentration in roots of plants grown on amended soil was significantly higher than in those of control plants. PASP, in this case, was the more effective chelant, since it increased the metal concentration almost three-fold above control levels. Again these data partially contradict the conclusions drawn by Komarek et al. (2010) regarding the limited Cu phytoextraction capacity of poplar, probably because they did not use a poplar clone specifically selected for this purpose. They do however corroborate our evidence regarding the outstanding phytostabilisation capacity of AL35, which can be further enhanced by a specific biodegradable chelant, such as PASP.

In general, a plant species can be considered a good candidate for phytoextraction purposes when, for a given metal, the TF > 1, otherwise, it is considered suitable for phytostabilisation (Fitz and Wenzel, 2002; Rizzi et al., 2004). The comparison of root BAF with leaf BAF gives a further qualitative indication on the capacity to translocate the metals to above-ground parts of the plant expressed by TF values. In the case of Cu, PASP-treated poplar plants generally presented lower leaf BAFs and TFs than control and EDTA/EDDS treated plants, but a higher root BAF, in agreement with the higher root Cu concentrations. Although the highest TF value for Cu, obtained with EDTA/EDDS, was still lower than 1.00 (0.26), we would not exclude the use of AL35 clone for phytoextraction purposes due to the high biomass that white poplar usually produces. PASP improved Cu uptake by roots, but this metal was not efficiently translocated to the aerial parts of the plants, suggesting that this chelant is particularly suitable for the phytostabilisation of Cu.

In the case of Zn, neither EDTA/EDDS nor PASP had, compared to controls, any effect on metal concentrations in the leaves, at least up to about 70 days after chelant addition. These results are in contrast with those reported by Luo et al. (2006b) and Mohavedi et al. (2011) in the case of a monocotyledonous plant (maize) and could be partially explained by the fact that, in our polluted soil, Zn is naturally highly available (one-fourth of the total concentration), but also by the natural capacity of white poplar to efficiently translocate and compartmentalise Zn at the leaf level (Castiglione et al., 2009; Cicatelli et al., 2010; Todeschini et al., 2011). Consequently, the mechanism by which Zn is transported to the leaves may already be operating at its maximum potential under control conditions.

In fact, in our study, the TF for this metal was lower, relative to controls, in chelant-amended soil. This can be attributed to the fact that, while foliar concentrations remained unaltered, those of the roots increased following soil treatment. Although the concentration of Zn remains high in the roots, as well as in the leaves of AL35, due to the presence of PASP, the TF, however, is substantial (2.72), suggesting that this chelant is not as detrimental as EDTA/EDDS to Zn translocation towards the leaves.

Although DTPA-extractable Zn concentrations in the soil were unaffected by the addition of chelants, both EDTA/EDDS and PASP significantly improved Zn accumulation at the root level, as demonstrated by the higher root BAF values compared with those of the controls. Although the mechanism by which plant uptake is enhanced despite unchanged soil metal availability remains to be elucidated, these results suggest that PASP can be used as an effective chelating agent for Zn phytostabilisation, even if a certain degree of root-to-shoot translocation was still maintained ($TF > 2$).

4.2. Improved Cu, but not Zn, removal from contaminated soil by chelants

Finally, in order to evaluate the benefits of chelant-assisted phytoremediation of Cu and Zn using clone AL35 of *P. alba*, the variations of metal concentrations in the polluted soil must be considered.

We can assume, based on the literature data (Cooper et al., 1999; Kos and Lestan, 2003; Meighan et al., 2011), that the bioavailable fraction of Cu was enhanced by chelant supply, particularly in the case of EDTA/EDDS, but also of PASP, albeit to a lesser extent. This hypothesis was indeed supported by the observation of an increased concentration of the metal in the leaves of chelant-treated plants compared to controls. The fact that increased bioavailability of the metal due to chelating agents was not observable at the second and third sampling dates could be explained by a degradation of EDDS and PASP in the course of time. Tandy et al. (2006) reported, in fact, the almost complete disappearance of EDDS from amended soil after only 25–30 days. A considerable absorption of the metal by the AL35 plants had also occurred. The decreased total Cu concentration in the soil at the end of the experiment can likewise be explained by the high amounts of the metal removed by AL35 plants and accumulated in the roots. On the other hand, Zn did not show any significant variation in amended soil relative to the unamended one, both in terms of total and bioavailable concentration. This might depend on the fact that, even though leaves are the plant organs accumulating most Zn, they represent a limited percentage (less than 15%; Faccioto, personal communication) of the total poplar biomass. This is probably not sufficient for an efficient soil phytoremediation.

5. Conclusions

The addition of chelants to this long-term heavily Cu- and Zn-contaminated soil improved the uptake of these HMs by the plants, and, depending on the metal, favoured their phytoextraction and/or phytostabilisation. While EDTA/EDDS, but not PASP, enhanced the phytoextraction of Cu, PASP instead increased root uptake of this HM, favouring its phytostabilisation. Given that EDTA/EDDS can have undesirable side effects and that PASP improved Cu and Zn uptake at the root level to the same extent as, or even better than, the chelant mixture, the highly biodegradable and non-toxic poly-amino acid can be a good choice for chelant-enhanced phytostabilisation. Moreover, PASP is considered one of the most promising compounds of the “green chemistry”, and its large-scale production is a well-established and cost-effective industrial process. For this reason, it is already used in agriculture to

improve crop yield. Further studies should be directed to improve the capabilities of PASP to solubilise HMs in order to use it in future field trials on polluted sites. Such trials are important because the success of this soil clean-up technology is strongly dependent, upon the soil's physical, chemical and biological characteristics, as well as the metal and the plants employed.

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