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*Improving phytoremediation efficiency
in metal-polluted wastes*

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Riassunto

Le attività civili, industriali e agricole sono responsabili del rilascio di inquinanti nell'ambiente. Tra le diverse fonti, la contaminazione del suolo da parte di metalli e metalloidi è una problematica di grande attualità. Contestualmente, la legislazione in materia di gestione e protezione ambientale di molti Paesi attribuisce alle tecnologie di bonifica biologiche, meno costose e di minor impatto ambientale, una rinnovata importanza, al pari dei più invasivi metodi di depurazione fisico-chimici.

Il presente studio riguarda il primo tentativo in Italia di applicazione della fitodecontaminazione *in situ* di un'area – sita a Torviscosa, Udine – marcatamente inquinata da metalli e inclusa nell'elenco nazionale dei siti prioritari per la bonifica (D.M. 468/2001). A partire dagli anni Trenta e per diversi anni, nel sito sono state scaricate ceneri di pirite derivanti dall'arrostimento del minerale pirite per l'estrazione dello zolfo. Le ceneri sono risultate marcatamente contaminate da vari metalli come arsenico, cadmio, rame, piombo e zinco. A termine del periodo industriale, lo scarto industriale è stato ricoperto con uno strato di 0,15 m di terreno di riporto non inquinato ma ricco in scheletro. A causa delle particolari caratteristiche negative del substrato, struttura fisica alterata, carenza di nutrienti e delle sfavorevoli condizioni idrologiche, la flora spontanea che ha colonizzato negli anni il sito è risultata essere rada e inadeguata allo svolgimento di un efficiente processo fitoestrattivo.

La realizzazione dell'impianto di fitoestrazione con specie da biomassa ha richiesto una cura particolare nella gestione del suolo e un adeguamento delle tecniche di coltivazione. In merito alla lavorazione del terreno, la coltivazione delle piante è avvenuta dopo aratura ed in comparazione con tecniche di ripuntatura, rispettivamente al fine di diluire le ceneri con il terreno di riporto e di mantenere uno strato superficiale non inquinato.

In situ sono state coltivate specie arboree (*Populus alba* L., *P. nigra* L., *P. tremula* L., *Salix alba* L.) ed erbacee di interesse agrario (*Helianthus annuus* L., *Lolium multiflorum* Lam., *Medicago sativa* L., *Raphanus sativus* L. var. *oleiformis* Pers.), valutando la

produzione di biomassa, l'accumulo di metalli ed il possibile ruolo dell'apparato radicale. In generale, un maggiore accrescimento radicale (lunghezza) è risultato correlato positivamente alla concentrazione di metalli raggiunta nei tessuti epigei, ma non con la loro rimozione. Nelle ceneri, l'aratura ha peggiorato l'accrescimento radicale di quasi tutte le specie saggiate a causa della maggiore presenza (totale e biodisponibile) di metalli rispetto alla ripuntatura.

Nel sito di Torviscosa, l'asportazione di inquinanti è risultata generalmente modesta a causa della scarsa produttività vegetale, e ha riguardato quasi esclusivamente lo zinco. Pioppo e salice sono stati comunque in grado di stabilizzare una porzione considerevole di metalli pesanti a livello radicale, suggerendo che i sistemi SRC (Short Rotation Coppice) possano fornire anche un importante contributo nella fitostabilizzazione. Questa possibilità sembra invece essere meno realistica per le radici fibrose delle colture erbacee, a causa del loro intenso e rapido turnover. Si rende tuttavia necessaria una precisa quantificazione della produttività radicale e del tempo di ritenzione dei metalli nelle radici fittonanti.

I risultati ottenuti presso il Woolston New Cut Canal (Inghilterra, UK), parte integrante di questa tesi, indicano che l'ammendamento della matrice inquinata con piccole quantità di cemento (1%) o di carbonato di calcio (1%) può contribuire a stabilizzare diversi metalli pesanti. Questo ridurrebbe la dispersione atmosferica e il dilavamento degli inquinanti, permettendo allo stesso tempo alle piante di migliorare la produttività per effetto della minore frazione di metalli biodisponibili.

Tra le colture saggiate, il rafano (*Raphanus sativus* var. *oleiformis*) ha dimostrato un elevato grado di adattamento alle ceneri di pirite, raggiungendo un buon potenziale produttivo e interessanti asportazioni di metalli. L'interesse per questa specie è stato pertanto esteso anche a sperimentazioni in ambiente controllato per studiare l'applicazione di auxine, acidi umici e sostanze chelanti.

L'applicazione ripetuta al suolo di acido indolbutirrico (IBA) non ha fornito i risultati attesi avendo ridotto l'accrescimento vegetale e l'accumulo di metalli sia a 0,1 che 1 mg IBA kg⁻¹. L'ormone ha invece fatto incrementare la lunghezza radicale tramite applicazioni fogliari a 10 mg IBA L⁻¹. Stimolazione dell'accrescimento radicale e miglioramenti del bilancio fitoestrattivo sono stati ottenuti con modeste applicazioni al suolo (0,1 g kg⁻¹) di acidi umici (HA), verosimilmente per l'attività ormono-simile e del debole effetto chelante.

Dosi superiori di HA (1 g kg^{-1}), un'opzione estremamente costosa su larga scala, non sono risultate efficaci, a causa del prevalente effetto fitotossico.

L'applicazione di acido etilendiaminodisuccinico (EDDS), un chelante meno persistente e più facilmente biodegradabile del ben noto acido etilendiaminotetracetico (EDTA), è stata sperimentata sia in *Raphanus sativus* var. *oleiformis* che in *Brassica carinata* A. Braun, ma solo raramente si sono osservati miglioramenti della fitoestrazione. L'EDDS ha infatti sempre aumentato la concentrazione di metalli nei tessuti epigei, indipendentemente dalla dose ($2,5$ e 5 mmol kg^{-1}) applicata poco prima della raccolta, ma la riduzione di biomassa è stata più marcata. Un'altra strategia possibile di gestione del chelante, ovvero applicazioni ripetute a basso dosaggio durante il ciclo colturale ($1 \text{ mmol kg}^{-1} \times 5$ volte), è risultata meno efficace a causa della considerevole fitotossicità e dell'aumento incontrollato di metalli lisciviati. Questi risultati suggeriscono che l'EDDS, analogamente ad altri chelanti, possa essere applicato proficuamente e senza rischi ambientali una settimana prima della raccolta a dosi moderate ($2,5 \text{ mmol kg}^{-1}$), indipendentemente dalla specie vegetale considerata.

Si può concludere che la gestione di siti inquinati da metalli pesanti tramite coperture vegetali agrarie e forestali possiede indubbiamente un importante valore paesaggistico, ma solo raramente può diventare una strategia concreta in condizioni estreme come quelle causate dalle ceneri di pirite. In queste circostanze la fitostabilizzazione è un'opzione da valutare con attenzione ed in termini di complementarietà alla fitoestrazione. Una corretta scelta delle specie e il perfezionamento delle tecniche agronomiche, così come il miglioramento delle condizioni chimico-fisiche della matrice inquinata e l'individuazione di trattamenti specifici, possono tuttavia rendere queste tecnologie ecocompatibili più efficaci e applicabili su larga scala.

Summary

Human activities are responsible for the release of pollutants into the environment, and soil contamination by metals and metalloids is of primary concern. Currently, the laws covering environmental management and protection of several countries are focusing on the less expensive and more environmentally friendly biological methods of restoration, compared with the more invasive physico-chemical ones.

The present study regards the first attempt in Italy to apply *in situ* phytoremediation of a metal-polluted, markedly degraded area located at Torviscosa (Udine, NE Italy) and included in the national priority list of sites for restoration. Within the site, pyrite cinders, derived from mineral roasting for sulphur extraction, had been severely contaminated by various metals such as arsenic, cadmium, copper, lead and zinc. The wastes were discharged in the Thirties and covered with an unpolluted 0.15-m layer of gravelly soil. Because of the particular substrate composition, poor physical structure, nutrient deficiency, and extreme hydrological conditions, the site was colonised by a rare and inadequate vegetation cover. Application of phytoremediation with biomass species required particular care in soil management and adjustment of the cultivation method. In this regard, ploughing was compared with subsoiling, in order to dilute the cinders with the unpolluted top layer and to maintain a shallow favourable layer for plant establishment, respectively.

In situ, trials were carried out to compare woody species (*Populus alba* L., *P. nigra* L., *P. tremula* L., *Salix alba* L.) and crops (*Helianthus annuus* L., *Lolium multiflorum* Lam., *Medicago sativa* L., *Raphanus sativus* L. var. *oleiformis* Pers.) in terms of above-ground productivity and metal accumulation, and to establish the role of root colonisation. Greater root lengths were generally correlated with metal concentrations in plant tissues but not with mass removals. The excessively high metal concentrations, both total and DTPA-extractable, of the wastes in ploughing cause worse rooting than subsoiling in almost all species.

In both woody and crop species, the mass balance of phytoextraction mainly involved Zn and was relatively poor because of generalised impaired growth. However, poplar and willow were able to stabilise a considerable fraction of trace elements at below ground, making the application of phytostabilisation (coarse roots) through short-rotation coppicing a reliable complementary strategy. Instead, this possibility seems less practicable in herbaceous crops through their finer roots, although more precise quantification of root production and turnover is required for insights into long-term metal stabilisation in tap-rooted species.

Results obtained at the Woolston New Cut Canal in the UK, which are an integral part of this thesis, indicate that stabilisation of several metals can also be achieved by adding soil with a small percentage of cement (1%) or lime (1%). This would reduce risks of metal movements through windblow and leachate transfer, and allow plants to improve their above-ground productivity.

Among crops, *Raphanus sativus* var. *oleiformis* showed the highest tolerance to pyrite wastes, providing interesting phytoextraction balances, thanks to less altered growth rather than increased metal concentrations. The consistency of these results with past experiments in another polluted site suggested that this species can be conveniently exploited by testing various methods of improvement in metal uptake (e.g., auxins, humic acids, chelators).

In pot trials, repeated soil applications of indolebutyric acid (IBA) have shown detrimental effects on plant growth and phytoextraction, regardless of doses of 0.1 and 1 mg kg⁻¹. These negative effects were not observed for foliar spraying at 10 IBA mg L⁻¹. Conversely, significant increases in root length and metal removal were seen after soil application of humic acids (HA) at small doses (0.1 g HA kg⁻¹ of substrate), probably because of their hormone-like and chelating (weak) effects. Higher doses of HA (1 g kg⁻¹), an extremely expensive option on a large scale, were fortunately not effective, as worsening of shoot biomass greatly exceeded improvements in metal concentrations.

The application of ethylene-diamine-disuccinic acid (EDDS), a less persistent and more easily degraded chelator than the traditional ethylene-diamine-tetraacetic acid (EDTA), was experimented in both *Raphanus sativus* var. *oleiformis* and *Brassica carinata* A. Braun, but only occasionally were good phytoextraction results observed. EDDS always improved metal concentrations, regardless of doses of 2.5 and 5 mmol kg⁻¹ (applications

close to harvest), but biomass reduction was a prevailing effect. Repeated low-dosage applications of EDDS ($1 \text{ mg kg}^{-1} \times 5 \text{ times}$) during the cycle, another possible strategy of chelator management, was less effective because of considerable phytotoxicity and undesirable marked increases in metal leaching. Like those found for other chelators, these results suggest that, for maximum effectiveness and absence of environmental risks, EDDS should be applied one week before harvest at a moderate dose (2.5 mmol kg^{-1}), regardless of species.

It is concluded that phytomanagement of metal-polluted sites with field crops and coppices undoubtedly has a landscape value, but may only seldom become a reliable phytoextraction strategy in extreme contamination conditions like those due to pyrite wastes. In these circumstances, phytostabilisation is an option which should be evaluated more carefully as additional complementary mean. For both phytoextraction and stabilisation, the proper choice of species and adjustment of its cultivation method, as well as substantial soil amelioration and identification of specific treatments, can significantly improve these green technologies.

Chapter I. Field crops for phytoremediation of metal-contaminated land. A review¹

Abstract

The use of higher plants to remediate contaminated land is known as *phytoremediation*, a term coined already 15 years ago. Among green technologies addressed to metal pollution, phytoextraction has received increasing attention starting from the discovery of hyperaccumulator plants, which are able to concentrate high levels of specific metals in the above-ground harvestable biomass. The small shoot and root growth of these plants, and the absence of their commercially available seeds has stimulated study on biomass species, including herbaceous field crops.

We review here the results of a bibliographic survey from 1995 to 2009 in CAB abstracts on *phytoremediation* and *heavy metals* for crop species, citations of which have greatly increased, especially after 2001. Apart from the most frequently cited *Brassica juncea* (L.) Czern., which is often referred to as an hyperaccumulator of various metals, studies mainly focus on *Helianthus annuus* L., *Zea mays* L., and *Brassica napus* L., the last also having the greatest annual increase in number of citations.

Field crops may compensate their low metal concentration by a greater biomass yield, but available data from *in situ* experiments are currently very few. The use of amendments or chelators is often tested in the field to improve metal recovery, allowing above-normal concentrations to be reached. Values for Zn exceeding 1,000 mg kg⁻¹ are found in *Brassica* spp., *Phaseolus vulgaris* L. and *Zea mays*, and Cu higher than 500 mg kg⁻¹ in *Zea mays*,

¹ From: Vamerali T., Bandiera M., Mosca G. (2009) Field crops for phytoremediation of metal-contaminated land. A review. Environ. Chem. Lett., DOI 10.1007/s10311-009-0268-0

Phaseolus vulgaris and *Sorghum bicolor* (L.) Moench.. Lead greater than 1,000 mg kg⁻¹ is measured in *Festuca* spp. and various *Fabaceae*. Arsenic has values higher than 200 mg kg⁻¹ in sorghum and soybean, whereas Cd concentrations are generally lower than 50 mg kg⁻¹.

Assisted phytoextraction is currently facilitated by the availability of low-toxic and highly degradable chelators, such as EDDS and nitrilotriacetate. Currently, several experimental attempts are being made to improve plant growth and metal uptake, and results are being achieved from the application of organic acids, auxins, humic acids and mycorrhization.

The phytoremediation efficiency of field crops is rarely high, but their greater growth potential compared with hyperaccumulators should be considered positively, in that they can establish a dense green canopy in polluted soil, improving the landscape and reducing the mobility of pollutants through water, wind erosion, and water percolation.

Introduction

Worldwide, soil is being seriously degraded as a result of increasing industrial, agricultural and civil activities. Soil contamination, both diffuse and localised, can lead to damage to several soil functions and contamination of surface and ground-water. The main sources of diffuse soil contamination is deposition from the atmosphere and flowing water or eroded soil itself. Further contamination may derive from direct application of pesticides, sewage sludge, fertilisers and manure, which often contain heavy metals. The soil functions most affected by contamination are buffering, filtering and transforming capacities (EEA, 2003).

Localised contaminated soils, currently called *brownfields* (French et., 2006) or *urban soils*, are frequently associated with abandoned industrial plant, accidental release of

pollutants, or inappropriate municipal and industrial waste disposal (EEA-UNEP, 2000). In the mining industry, which is a major cause of soil degradation, the risk of contamination is associated with sulphur- and metal-bearing tailings stored in mining sites, and the use of chemicals such as cyanide in the refining process (EEA, 2003).

Among various organic and inorganic pollutants, great worldwide concern about soil contamination regards heavy metals. In the European Union, contamination by metals accounts for more than 37% of cases, followed by mineral oil (33.7%), Polycyclic Aromatic Hydrocarbons (PAH, 13.3%), and others (EEA, 2007).

Many industrialised countries are now focusing on regulations to reduce the impact of pollution. For instance, the European Pollutant Release and Transfer Register (Regulation 166 EC, 2006) lists possibly dangerous activities and pollutants, and provides thresholds for releases into air, water and soil for all main contaminants. As regards metals, the maximum amounts permitted are generally higher for air than for water or soil (Table 1).

Table 1. Thresholds for release of inorganic pollutants to air, water and land in a single site. All metals are considered as total mass of element in all chemical forms (from Annex II, Regulation 166/2006/EC).

Pollutants and their compounds	Threshold for release (kg year ⁻¹)		
	To air	To water	To land
Arsenic	20	5	5
Cadmium	10	5	5
Chromium	100	50	50
Copper	100	50	50
Mercury	10	1	1
Nickel	50	20	20
Lead	200	20	20
Zinc	200	100	100

The most highly developed remediation methods for metal-contaminated soils are physical or chemical, such as soil washing, excavation and reburial. *Phytoremediation*, which uses plants to take up metals, is a cheap alternative technology, which is solar-driven and performed directly *in situ* (Salt et al., 1998). Removing heavy metals through harvestable biomass is an efficient technique for inorganic pollutants. Plants used for this purpose should ideally combine high metal accumulation in shoots and high biomass

production. Starting from the discovery of hyperaccumulator plants, which are able to concentrate high levels of specific metals in the above-ground biomass, there is now great interest in crop species which may solve the problem of the small biomass of hyperaccumulators. In this review, we present a summary of results from *in situ* experiments carried out with field crops in metal-contaminated soils.

Heavy metals in soils and plants

The term *heavy metal* generally refers to a specific group of elements with metallic properties (metals and semimetals), often associated with contamination and potential toxicity or ecotoxicity (Duffus, 2002). Over the past two decades, this term has been used increasingly in the literature and in legislation related to chemical hazards and the safe use of chemicals. From the bibliographic survey made by Duffus (2002), the term *heavy metals* appears to be commonly applied to elements of density higher than $3.5\text{-}7\text{ g cm}^{-3}$ and high atomic number (higher than 20), and includes transition metals, some metalloids, lanthanoids and actinoids.

Although some metals are essential for plant and animal life, many are toxic at high concentrations, and awareness of the extent and severity of soil and water contamination they cause is growing. Besides their natural availability in soils, specific sources of heavy metals are mine tailings, leaded gasoline and lead-based paints (The Conservation Foundation, 1987; Pirbazari et al., 1989), fertilisers, animal manure, biosolids, compost, pesticides, coal combustion residues, and atmospheric deposition (Adriano, 2001).

Metal(loid)s of environmental concern are As, Cd, Cr, Cu, Pb, Hg, Ni, Se, Mo, Zn, Tl, Sb, and others (Basta et al., 2005). Their anthropogenic application to soils is often related to the use of residuals, like biosolids, livestock manure and compost, adversely affecting human, crop and wildlife health (Adriano, 2001). In plants, some metals play an

important role as micro-nutrients, being essential for growth at low concentrations. Most of them are cofactors of enzymes and are involved in important processes such as photosynthesis (Mn, Cu), DNA transcription (Zn), hydrolysis of urea into carbon dioxide and ammonia (Ni), and legume nodulation and nitrogen fixation (Co, Zn, Co). Some are involved in flowering and seed production and in plant growth (Cu, Zn), especially when their availability is very low (Table 2). Interactions for uptake and transport may occur among metals or with macro-nutrients, depending on their relative concentrations. For instance, Cu reduces the uptake of Cd and Ni in soybean seedlings (Cataldo and Wildung, 1978), whereas its uptake is inhibited by Cr, Cd, Co and Ni in barley. Nickel can compete with Cu, Zn and Co and, to a greater extent, with iron uptake (Cataldo et al., 1978). Lead is also an antagonist in the uptake of Fe, more than Mn and Co (Gaweda and Capecka, 1995) and can inhibit enzymes such as ureases. In rice, the competition in uptake between arsenate and phosphate, which may markedly reduce plant growth, is well-known (Meharg and Hartley-Whitaker, 2002). Interactions among metals for uptake across cellular membranes and vacuoles and transport depend on the expression and functionality of specific transporter families shared by various metals (Hall and Williams, 2003).

Phytotoxicity is mainly associated with non-essential metals like As, Cd, Cr and Pb, which generally have very low toxicity thresholds (Clemens, 2006) and lower values for hyperaccumulation (especially for Cd) (see below) than the other metals. The above-mentioned metals, except Cr, are also not essential for humans, and may enter the food chain through ingestion of contaminated edible products at various levels, depending on the metal in question. Arsenic, Cr and Pb are not easily transferred to above-ground plant biomass, mainly being stored in root cells (Marin et al., 1992, Tiwari et al., 2009, Mellem et al., 2009), whereas Zn is easily accumulated in green tissues like leaves (Probst et al., 2009).

Uptake of metals is mainly influenced by their bioavailable fraction rather than by the total amount in soil. Although the low availability of soluble forms of metals is desirable for food production, for phytoextraction the opposite is required, for better efficiency in soil remediation. Metal availability depends on: i) the intensity of adsorption to soil particles; ii) the ability of plants to desorb and transfer metals to their tissues; (iii) interactions with soil micro-organisms (Salt et al., 1998; Lasat, 2002).

Bioavailable metals include a water-soluble fraction, which is in equilibrium with cation exchange sites of soil organic matter or clays, including forms chelated to inorganic or organic soil constituents (MacCarthy, 2001). In general, both high cation exchange capacity (CEC) and soil pH reduce metal bioavailability, and thus mobility and possible leaching. Conversely, the anionic behaviour of As leads to its greater mobility in conditions of high pH and organic matter and reduced contents of iron and oxygen in soil (Hartley et al., 2009).

Organic matter forms metal complexes, so that it can either reduce metal mobility or increase availability when the complexes are soluble in the soil solution (Halim et al., 2003). The redox potential of soil is directly involved in metal availability, as anaerobic conditions generally induce precipitation of sulphides (Cd, Co, Cu, Ni, Pb, Sn, Zn), with the exception of Mn, which increases its availability (Suthersan, 1997).

Within a certain interval of contamination, plants are able to defend themselves from heavy metals. Baker and Walker (1989) classified plants in three groups, excluders, indicators and accumulators, the concentrations of pollutants in shoot biomass being respectively lower, similar and higher than that of the soil. When great amounts of metals enter plants, various mechanisms of detoxification are possible, such as reduced translocation (Angelova and Ivanov, 2009), compartmentalisation (vacuoles, cell walls) (Lasat et al., 1998), chelation with phytochelatins (Cobbett, 2000), and bio-transformation (Tomsett et al., 1992).

Table 2: Role and toxicity of metals in plants and humans. Hyperaccumulator plant species refer to temperate climates. Variations in phytotoxicity thresholds take into account interspecific and environmental variability.

(a) Wallace et al., 1980; (b) Förstner, 1995; (c) Li et al., 2009; (d) McCutcheon and Schnoor, 2003; (e) Terry and Bañuelos, 2000; (f) Visoottiviseth et al., 2002; (g) Zayed and Terry, 2003; (h) Adriano, 1986.

Metal	Essential for plants	Functions in plants	Toxicity threshold in plant tissues (mg kg ⁻¹ d.w.)	Some hyperaccumulators	Threshold for hyperaccumulation above-ground biomass (mg kg ⁻¹ d.w.)	Essential for human	Toxicity symptoms in humans
As	No	-	>~20 (a)	<i>Pteris vittata</i> L. (f)	>1,000 (f)	No	Cancer (e.g. lung and skin); cardiovascular, gastrointestinal, hepatic and renal diseases
Cd	No	-	5-10 (b)	<i>Thlaspi caerulescens</i> J. & C. Presl. (e)	>100 (e)	No	Stomach irritations (vomiting and diarrhoea); lung damage; kidney diseases; cancer (probably)
Co	Yes	Cofactor of biosynthetic enzymatic activities; essential for <i>Rhizobium</i>	60-170 (c)	<i>Haumaniastrum robertii</i> (Robyns) P.A.Duvign. & Plancke (e)	>1,000 (e)	Yes	Contact dermatitis; mutagenic and carcinogenic effects
Cr	No	-	1-2 (b)	<i>Brassica juncea</i> (L.) Czern.; <i>B. napus</i> (L.); <i>Vallisneria americana</i> (d)	>20,000 (g)	Yes	Genotoxic carcinogens (Cr ⁶⁺); lung cancer

Table 2. (Continued).

Metal	Essential for plants	Functions in plants	Toxicity threshold in plant tissues (mg kg ⁻¹ d.w.)	Some hyperaccumulators	Threshold for hyperaccumulation in above-ground biomass (mg kg ⁻¹ d.w.)	Essential for human	Toxicity symptoms in humans
Cu	Yes	Constituent of enzymes; role in photosynthesis; involved in reproductive and in determining yield and quality in crops	15-20 (b)	<i>Brassica juncea</i> (L.) Czern.; <i>Vallisneria americana</i> Michx. (d)	>1,000 (e)	Yes	Inhibition of dihydrophil hydratase (in haemopoiesis); accumulation in liver and kidney
Mn	Yes	Constituent of enzymes; activation of enzymes; photosynthesis; reproductive phase; resistance against biotic and abiotic stress	170-2,000 (h)	<i>Agrostis castellana</i> Boiss. & Reuter (d)	>10,000 (e)	Yes	Neurological symptoms; affection of liver function
Ni	Yes	Constituent of enzymes; activation of urease	20-30 (b)	<i>Alyssum</i> spp.; <i>Thlaspi</i> spp. (e)	>1,000 (e)	Yes	Accumulation in lungs

Table 2. (Continued).

Metal	Essential for plants	Functions in plants	Toxicity threshold in plant tissues (mg kg ⁻¹ d.w.)	Some hyperaccumulators	Threshold for hyperaccumulation in above-ground biomass (mg kg ⁻¹ d.w.)	Essential for human	Toxicity symptoms in humans
Pb	No	-	10-20 (b)	<i>Thlaspi rotundifolium</i> (L.) Gaudin ssp. <i>Cepaeifolium</i> (Wulfen) Rouy & Fouc; <i>T. caerulescens</i> J. & C. Presl.; <i>Alyssum wulfenienum</i> Bernh.; <i>Arrhenatherum elatius</i> (L.) Beauv.; <i>Festuca ovina</i> L. (e)	>1,000 (e)	No	Irreversible neurological damage; renal disease; cardiovascular effects; reproductive toxicity
Zn	Yes	Constituent of cell membranes; activation of enzymes; DNA transcription; involved in reproductive phase and in determining yield and quality of crops; resistance against biotic and abiotic stress; legume nodulation and nitrogen fixation	150-200 (b)	<i>Thlaspi</i> spp.; <i>Cardaminopsis</i> spp. (e)	>10,000 (e)	Yes	Inhibition of copper absorption; nausea, vomiting, loss of appetite, abdominal cramps, diarrhoea, headaches

In general, extensive root colonisation is essential for metal uptake, as it has been widely shown for nutrients in several plant species. Rhizosphere acidification and release of root exudates contribute to the absorption of several heavy metals (Lasat, 2002), as reported in *Graminae* specifically for iron (Kanazawa et al., 1994) and zinc (Cakmak, 1996). Exudates may also be involved in the mechanisms of plant tolerance to heavy metals (Pellet et al., 1995; Larsen et al., 1998). Some root morphological traits, such as pattern of root density, maximum depth and specific root length, are considered crucial for adaptation to stress conditions (Fitter and Stickland, 1991). Phenotypic root plasticity enables plants to cope with a wide range of soil factors and heterogeneity of soils. In this regard, dicotyledons exhibit greater plasticity than grasses (Eissenstat, 1992; Taub and Goldberg, 1996). Higher root proliferation is usually observed in favourable (e.g., fertilised) micro-sites, but root response also depends on the mobility of nutrients (Campbell and Grime, 1989). As regards heavy metals, it has been noted that hyperaccumulator plants (e.g., *Thlaspi caerulescens* J. & C. Presl.) have preferential root growth in the zones where the metals are present (Schwartz et al., 1999; Whiting et al., 2000), thus increasing the efficiency of remediation in soils heterogeneously contaminated.

Phytoremediation

The term *phytoremediation*, from the Greek *phyto*, meaning “plant”, and the Latin suffix *remedium*, “able to cure” or “restore”, was coined by Ilya Raskin in 1994, and is used to refer to plants which can remediate a contaminated medium. Phytoremediation takes advantage of the plant's ability to remove pollutants from the environment or to make them harmless or less dangerous (Raskin, 1996). It can be applied to a wide range of organic (Anderson and Coats, 1995; Schnoor et al., 1995) and inorganic contaminants.

Phytoremediation is a general term including several processes (Table 3), among which phytoextraction and phytostabilisation are the most reliable for heavy metals.

Table 3: Differing areas of phytoremediation (from Salt et al., 1998; Dietz and Schnoor, 2001).

Technology	Description
Phytoextraction	Uptake of pollutants from environment and their concentration in harvestable plant biomass
Phytostabilisation	Reduction of mobility and bioavailability of pollutants in environment
Phytovolatilisation	Removal of pollutants from soil or water and their release into air, sometimes as a result of phytotransformation to more volatile and/or less polluting substances
Phytotransformation	Chemical modification of pollutants as a result of plant metabolism, both <i>in planta</i> and <i>ex planta</i> , often resulting in their inactivation, degradation (phytodegradation) or immobilisation (phytostabilisation)
Rhizofiltration	Use of plant roots to absorb and adsorb pollutants or nutrients from water and wastewater (e.g., buffer strips)

Metal phytostabilisation

Phytostabilisation does not aim at removing contaminants from the soil, but at reducing their risks to human health and the environment. The establishment of a green canopy in polluted soil has the effect of reducing the mobility of pollutants through water, wind erosion and water percolation. A significant fraction of metals can be stored at root level, especially in polyannual species (Vamerali et al., 2009) contributing to long-term stabilisation of pollutants. Much literature refers to phytostabilisation as a way of reducing mobility and excluding metals from plants. Application of soil amendments, such as phosphate fertilisers, organic matter, Fe- and Mn-oxydrides and inorganic clay minerals, contributes to integrating the role of plants by reducing metal bioavailability, and thus preventing both plant uptake and leaching (Berti and Cunningham, 2000). However, root sequestration of metals is not definitive, as removal of pollutants from the environment may last after tissue degradation (Vangronsveld et al., 1995; Arienzo et al., 2004).

Phytostabilisation improves the chemical and biological characteristics of contaminated soil by increasing the amount of organic matter, nutrient levels, cation exchange capacity and biological activity (Arienzo et al., 2004). In several cases, a vegetation cover has been found to provide a cost-effective and environmentally sustainable method of stabilising and reclaiming toxic metal mine sites (Mench et al., 2003; Wong, 2003).

Although this technique is effective in remediating metal-contaminated soils and sediments, King et al. (2006) showed that it fails when applied, for instance, to canal sediments because of the high mortality of various species such as poplars, willows and alders. According to Sutton and Dick (1987), soil acidity is the main constraint for the establishment of vegetation in these environments, although the application of organic residues (slight effect) and liming materials (Ye et al., 1999; Wong, 2003) or lime-stabilised biosolids can attenuate the effects (Abbott et al., 2001; Basta et al., 2001; Adriano et al., 2004), allowing a vegetation cover to form (Brown et al., 2003; Brown et al., 2005).

The choice of plant species is an important task in a phytostabilisation-based technique (Rizzi et al., 2004). Plants must be able to develop extended and abundant root systems and keep the translocation of metals from roots to shoots as low as possible (Mendez and Maier, 2008).

Metal phytoextraction

As heavy metals are the main inorganic contaminants, among existing phytotechnologies much interest is devoted to phytoextraction and its improvement (Adriano, 1986, 1992; Alloway, 1990; Meeuseen et al., 1994). Phytoextraction is a *green* technology, born 15 years ago from the studies of Raskin (1994) and later of Brooks et al. (1998), which exploits the ability of plants to translocate a great fraction of metals taken up to harvestable biomass. Contaminated biomass may be used for energy production, whereas remaining ashes are dumped, included in construction materials, or subjected to metal extraction (phytomining) (Brooks et al., 1998).

Although promising, phytoextraction has many limitations, deriving from scarce metal availability in soils, difficulties in root uptake, symplastic mobility and xylem

loading, as well as the great energy cost required for detoxification and storage within shoots (Meagher, 2000; Clemens et al., 2002).

Plants show differing morpho-physiological responses to soil metal contamination. Most are sensitive to very low concentrations; others have developed tolerance, and a reduced number show hyperaccumulation (Baker and Brooks, 1989; Barceló et al., 1994; Brooks, 1998). The latter capacity has practically opened up the way to phytoextraction (Garbisu and Alkorta, 2003; Van der Lelie et al., 2001).

Metal accumulation is expressed by the metal biological absorption coefficient (BAC), i.e., the plant (harvestable)-to-soil metal concentration ratio (Blaylock et al., 1997). Besides convenient BAC, both the high bioconcentration factor (BCF, root-to-soil metal concentration ratio) and the translocation factor (TF, shoot-to-root metal concentration ratio) can positively affect phytoextraction. Tolerant plant species tend to restrict soil-root and root-shoot transfers, and therefore have much less accumulation in biomass, whereas hyperaccumulators actively take up and translocate metals into above-ground tissues. Plants with high BAC (greater than 1) are suitable for phytoextraction; those with high BCF (higher than 1) and low TF (lower than 1) have potential for phytostabilisation (Yoon et al., 2006).

Desirable characteristics for a plant species in phytoextraction are: (i) fast growth and high biomass; (ii) extended root system for exploring large soil volumes; (iii) good tolerance to high concentrations of metals in plant tissues; (iv) high translocation factor; (v) adaptability to specific environments/sites; (vi) easy agricultural management. All these traits are difficult to combine, and there are basically two available phytoextraction strategies, which make use of hyperaccumulators or biomass plant species, respectively. Hyperaccumulators, such as the well-studied *Thlaspi caerulescens* J. & C. Presl. and *Alyssum bertolonii* Desv. (McGrath, 1998; Robinson et al., 1998) are able to take up specifically one or a few metals, generally producing a small shoot biomass with high metal concentrations (Baker and Brooks, 1989; Reeves and Baker, 2000). Instead, high-yielding biomass plant species can absorb a wide range of heavy metals at generally low concentration.

3.2.1 Hyperaccumulator plants

Hyperaccumulation is an unusual occurrence, ascertained in a narrow range of species which often grow in metal-rich sites, like serpentine and calamine (Brooks et al., 1977). By analysing the metal contents of several species from worldwide collections, Baker and Brooks (1989) suggested the following values of metal concentrations in shoots for hyperaccumulation without evident symptoms of toxicity: 100 mg kg⁻¹ for Cd, 1,000 mg kg⁻¹ for Ni, Cu, Co and Pb, and 10,000 mg kg⁻¹ for Zn and Mn (Table 2). Although these threshold values were fixed arbitrarily, they roughly lie at least at one order of magnitude greater than those found in common species (Salt and Kramer, 2000). Hyperaccumulators are generally minor vegetation components in most European and North American habitats, but are sometimes relatively abundant in some locations in New Caledonia, Cuba and South Africa. Currently, more than 400 hyperaccumulator species are known, belonging to 45 different botanical families (Salt et al., 1998), among which the most frequent are *Brassicaceae*, with the genus *Thlaspi*, *Alyssum* and *Brassica* (e.g., *Thlaspi caerulescens* J. & C. Presl., *T. rotundifolium* (L.) Gaudin, *Brassica juncea* (L.) Czern, *Cardaminopsis* spp., *Alyssum* spp.), and *Fabaceae* (Baker et al., 2000). Among monocotyledons, *Poaceae* includes, for instance, *Agrostis castellanea* Boiss. & Reut., *Arrhenatherum elatius* (L.) Beauv., *Festuca ovina* L. as hyperaccumulators (Prasad and De Oliveira-Freitas, 2003). The habitus of these plants varies from small annual herbaceous plants to perennial shrubs and trees, although they mainly occur as grasses in temperate climates. A few hyperaccumulator trees are found in New Caledonia, with *Psychotria douarrei* Beauv. accumulating up to 27,700 mg Ni kg⁻¹ in its shrubs (Boyd et al., 1999) and *Sebertia acuminata* Pierre (*Rubiaceae*), which produces a latex with an extremely high Ni concentration (up to 26% in dry mass) (Jaffre et al., 1976). One of the most frequently investigated species is *Thlaspi caerulescens* J. & C. Presl., a hyperaccumulator of Zn and Cd (McGrath et al., 2006), which has often been studied for better understanding of the mechanisms of metal tolerance and for gene manipulation (Dushenkov et al., 2002; Guan et al., 2008).

Lack of information on the agricultural management of hyperaccumulators, together with slow-growing and poor shoot and root growth, increase the difficulties in the practical application of these species in remediation projects (Navari-Izzo and Quartacci, 2001; Lasat, 2002). There is also little knowledge on their rooting. In the case of *Thlaspi*

caerulescens J. & C. Presl., preferential root development in soil regions where heavy metals are more concentrated was found to be a favourable trait for remediating heterogeneously contaminated soils (Schwartz et al., 1999; Whiting et al., 2000). Although hyperaccumulators have the advantage of tolerating high soil concentrations of specific contaminants, when further metals severely pollute the soil they die, like common species, as found for *Thlaspi caerulescens* J. & C. Presl. and *Haumaniastrum robertii* (Robyns) P.A. Duvign. & Plancke in a pluricontaminated soil (Quartacci et al., 2003). The major limitations in application of phytoremediation involve the most phytotoxic metals, like Cd, Pb and Cr (Nanda-Kumar et al., 1995, Blaylock et al., 1997, Huang et al., 1997). Fast-growing herbaceous hyperaccumulators have been identified for Ni, with *Alyssum bertolonii* Desv. and *Berkheya coddii* Roessler able to reach 9 and 22 t ha⁻¹ of harvestable dry biomass, respectively (Robinson et al., 1997a; Robinson et al., 1997b). Relatively large biomass may also be obtained from the arsenic hyperaccumulator fern *Pteris vittata* L. when grown in favourable climates (Ma et al., 2001), reversing the generally negative correlation between plant biomass and metal concentration.

Crop species

The potential of some crops for phytoextraction purposes has been studied in the last few years (Baker et al., 1994; Ebbs and Kochian, 1997; Ebbs et al., 1997), especially within *Brassicaceae*, in view of the large number of hyperaccumulators belonging to this family. Interesting Pb accumulations at shoot level have been found in *Brassica* species (*B. nigra* (L.) Koch, *B. carinata* A. Braun, *B. oleracea* L., *B. campestris* L., *B. juncea* (L.) Czern., *B. napus* L.) up to 3.5% of dry weight (Nanda-Kumar et al., 1995). Herbaceous or woody biomass species may be promising in view of their high-yielding ability, which can compensate for low concentrations of contaminants in their tissues (Chaney et al., 1997), thus resulting in similar or even higher offtake of pollutants than hyperaccumulators.

In this paper, a bibliographic survey of the literature over the period 1995-2009 was carried out, in order to take a census of crop species involved in experimental research worldwide on *phytoremediation* and *heavy metals*, these terms being sought in CAB abstracts database regardless of search field (Table 4). In the last 14 years, the most frequently cited species was *Brassica juncea* (L.) Czern. (148 citations), followed by

Helianthus annuus L. (57), *Brassica napus* L. and *Zea mays* L. (both 39 citations). In the ranking, *Brassicaceae* (*Raphanus sativus* L., *Brassica carinata* A. Braun) and *Poaceae* (*Festuca* spp., *Lolium* spp., *Hordeum vulgare* L.) were again represented, whereas fewer citations were made of *Fabaceae* such as soybean, bean, alfalfa and pea. The greater interest in *Brassicaceae* derives from the fact that research on these species started earlier, together with the interesting concentrations they provide, especially for *Brassica juncea* (L.) Czern.. The latter has been described as a hyperaccumulator for various metals such as Cd, Cr, Cu, Mn, Pb, Se and Zn. Apart from phytoextraction of radioisotopes (Cs^{137}) (McCutcheon and Schnoor, 2003), the ability of sunflower to take up Cd, Pb and Zn has been reported by several authors (e.g., Fellet et al., 2007; Tassi et al., 2008). Maize was successfully experimented in phytoextraction of Cd and other metals such as Ni, Cu, Pb and Zn (Wu et al., 2007; Murakami and Ae, 2009), often in association with the use of chelators, mycorrhizae, bacteria and other devices, such as application of sulphur and co-planting with hyperaccumulators. Among the most represented families, interest focuses mainly on a few species for *Brassicaceae* and a greater number of *Poaceae*, which have been studied more recently, together with *Fabaceae*.

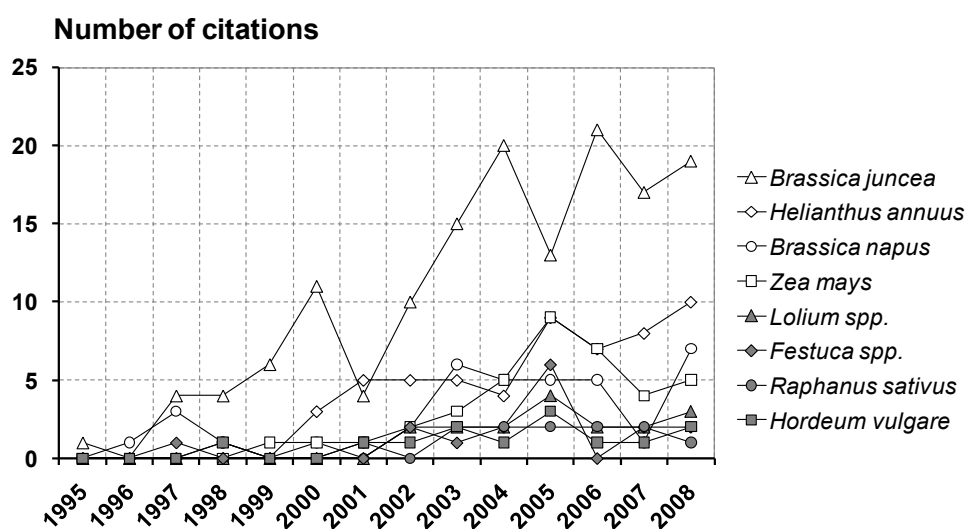


Figure 1. Dynamics of number of citations (CAB Abstracts database) over last 14 years for most frequently studied field crops in phytoremediation. Keywords (regardless of search field): *phytoremediation* and *heavy metals*.

Until now, research has been carried out continuously for almost all species, with an increasing rate of citations per year, which varies from 155% for oilseed rape to 3% for

maize (Figure 1), suggesting that this citation rate may be an index of species efficiency. Until now, most results come from hydroponic- or pot-greenhouse or laboratory experiments, making it difficult to transfer data to open-field conditions, although site-specific results are progressively increasing.

In an attempt to provide realistic results, Table 5 lists data from field and some pot experiments only. For field crops, data on metal concentrations are available mainly for Cu, Zn, Pb and Cd, which are frequently found in polluted sites; less information is available for As, and only a few experiments deal with Co, Cr and Ni. The use of amendments or chelators is often tested in the field to improve metal uptake, allowing above-normal concentrations to be reached. Among the most frequently studied metals, interesting Zn concentrations (higher than $1,000 \text{ mg kg}^{-1}$) are found in *Brassica* spp., *Phaseolus vulgaris* L. and *Zea mays* L. and Cu (above 500 mg kg^{-1}) in *Zea mays* L., *Phaseolus vulgaris* L. and *Sorghum bicolor* (L.) Moench. Concentrations of Pb greater than $1,000 \text{ mg kg}^{-1}$ are found in *Festuca* spp. and *Fabaceae* such as *Medicago sativa* L.. On the basis of the generally low concentrations, Cd phytoextraction for crops does not seem to be reliable, as contents of 20 mg kg^{-1} in maize and about 50 in alfalfa and bean were reached. Arsenic shows concentrations of more than 200 mg kg^{-1} in sorghum and soybean. Much of this literature reports impaired shoot growth (Clemente et al., 2005) and the need to use chelators to improve metal recovery (Luo et al., 2005, 2008). In many cases, organic amendments and fertilisation also aimed at increasing soil fertility (Marchiol et al., 2004) and soil pH (Clemente et al., 2005) in markedly degraded sites.

The use of field crops for phytoremediation purposes should not consider the use of products for animal feed or human consumption, although in many cases risks are small, since translocation to grains of various metals is limited. For instance, negligible Cd and Cu contents in maize kernels and oilseed rape seeds were found in a pluri-contaminated soil by Wang et al. (2002). We also found small concentrations of metals in sunflower achenes and maize kernels in field plots in a soil severely contaminated with Cd, Pb, Cu, Mn and Zn (Mosca et al., 2004). From these results, obtained without application of amendments, interesting Zn and Mn concentrations (Table 6) and total offtake (about 3 kg ha^{-1} for both metals) were found in sunflower and maize, but translocation to maize kernels was relatively poor (Mn: 12%; Zn: 18% of the above-ground contents) (Figure 2).

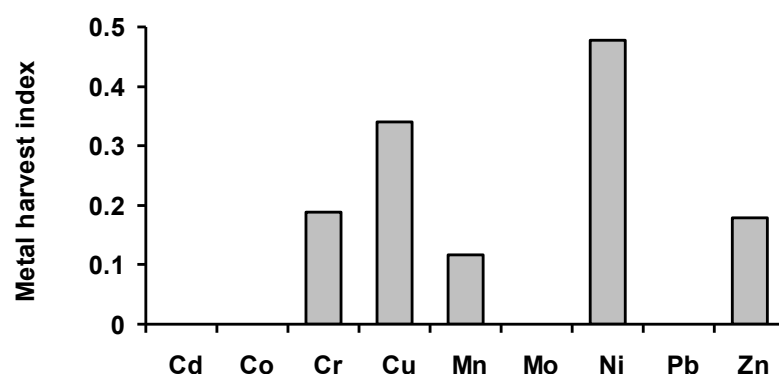


Figure 2. Fraction of above-ground metals accumulating in maize grain at harvest in a pluri-contaminated soil in Milan (Italy). Source: Mosca et al., 2004.

Table 4. Number of citations per crop species found in a bibliographic survey in CAB abstracts database over period 1995-2009 (until April). Regardless of database search field, keywords were: *phytoremediation* and *heavy metal(s)*.

Family	Species	No. of citations	Average rate of variation in citations (% year ⁻¹)	Period	
				From	To
<i>Asteraceae</i>	<i>Helianthus annuus</i> L.	57	+88	2000	2009
<i>Brassicaceae</i>	<i>Brassica juncea</i> (L.) Czern.	148	+10	1995	2009
	<i>Brassica napus</i> L.	39	+155	1996	2009
	<i>Raphanus sativus</i> L.	13	+39	1998	2008
	<i>Brassica carinata</i> A. Braun	11	+17	1997	2008
	<i>Sinapis alba</i> L.	4	-	2003	2007
<i>Fabaceae</i>	<i>Glycine max</i> (L.) Merr.	9	+10	2002	2007
	<i>Phaseolus vulgaris</i> L.	8	+5	2001	2008
	<i>Medicago sativa</i> L.	7	+18	2000	2007
	<i>Pisum sativum</i> L.	2	-	2003	2007
<i>Poaceae</i>	<i>Zea mays</i> L.	39	+3	1999	2009
	<i>Lolium spp.</i>	19	+22	2002	2009
	<i>Festuca spp.</i>	16	+15	1997	2008
	<i>Hordeum vulgare</i> L.	13	+16	2001	2008
	<i>Sorghum spp.</i>	9	+100	2002	2009
	<i>Triticum spp.</i>	9	+12	2000	2005
	<i>Avena sativa</i> L.	4	-	1998	2008
	<i>Oryza sativa</i> L.	2	-	2008	2009

Table 5. Metal concentrations in herbaceous crops from field/site experiments in recent bibliography (later than 2000). Possible treatments and phytotoxicity are listed.

Species	Metal concentrations (mg kg ⁻¹)								Treatments	Phytotoxicity	References
	As	Cd	Co	Cr	Cu	Ni	Pb	Zn			
<i>Brassica carinata</i> A. Braun	12	12	-	9.8	37	7.6	50	1,650		Yes	Marchiol et al., 2004; Soriano and Fereres, 2003
<i>Brassica juncea</i> (L.) Czern.	30	10	-	5.2	71	-	55	2,029	Cow manure (As, Cu, Zn) or mature compost (Pb)	Yes	Clemente et al., 2005; Marchiol et al., 2004
<i>Brassica napus</i> L.	5.8	11	-	9	40	7	39	1,400		No	Marchiol et al., 2004;
<i>Festuca</i> spp.	-	-	-	-	106	-	-	90			Álvarez et al., 2003
<i>Glycine max</i> (L.) Merr.	230	2.4	-	-	440	-	72	430		No	Fellet et al., 2007
<i>Helianthus annuus</i> L.	20	0.64	0.71	-	70	-	5	150	Mineral fertilisation (Co)	No	Fellet et al., 2007; Marchiol et al., 2007
<i>Hordeum vulgare</i> L.	20	0.44	-	-	16	-	27	334		-	Soriano and Fereres, 2003
<i>Lolium perenne</i> L.	-	-	-	-	29	-	140	59	Organic amendment	No	Alvarenga et al., 2009
<i>Medicago sativa</i> L.	85	53	-	-	77	-	2,177	-	EDTA (5mM) for Pb only	-	Pajuelo et al., 2007

Table 5. (Continued).

Species	Metal concentrations (mg kg ⁻¹)								Treatments	Phytotoxicity	References
	As	Cd	Co	Cr	Cu	Ni	Pb	Zn			
<i>Oryza sativa</i> L.	-	-	-	-	34	-	6	90		-	Murakami and Ae, 2009
<i>Phaseolus vulgaris</i> L.	-	53	-	-	2,230	-	1,000	1,440	Hot EDTA (Cd, Pb), EDDS (Cu, Zn)	-	Luo et al., 2005; Luo et al., 2008
<i>Pisum sativum</i> L.	-	-	-	-	-	-	1,390	-	EDTA	-	Chen et al., 2004
<i>Raphanus sativus</i> L.		9.4		5	34	6.5	28	1,450		Yes	Marchiol et al., 2004
<i>Sorghum bicolor</i> (L.) Moench	240	3.7	1.8	-	540	-	100	580	Pyrite cinders (Cd)	No	Fellet et al., 2007; Marchiol et al., 2007
<i>Triticum secalotriticum</i> Wittm.	21	1.9	-	-	27.5	-	37	588		-	Soriano and Fereres, 2003
<i>Zea mays</i> L.	30	20	-	-	1,220	-	257	1,200	EDDS (Cu, Zn) or EDTA (Cd, Pb)	No	Fellet et al., 2007; Luo et al., 2005

Table 6. Contents of metals in crops in a pluricontaminated site in Milan (Italy) (site experiment). Data refer to shoots (oilseed rape, linseed) and to all aboveground biomass ‘residues+grains’ (sunflower, maize, fodder radish) at harvest. Letters: comparison among species for same metal (LSD test, $P \leq 0.05$). *Source*: Mosca et al., 2004.

Metal	<i>Brassica napus</i> L. var. <i>oleifera</i> D.C.		<i>Helianthus annuus</i> L.		<i>Linum usitatissimum</i> L.		<i>Raphanus sativus</i> L. var. <i>oleiformis</i> Pers.		<i>Zea mays</i> L.	
Cd	1.19	c	2.27	b	5.04	a	1.19	c	0.40	d
Co	0.05	ab	0.08	a	-		0.01	b	-	
Cr	0.53	ab	0.58	a	0.48	ab	0.61	a	0.35	b
Cu	7.76	b	15.7	a	7.62	bc	5.26	c	5.15	c
Mn	103	ab	170	a	36.6	ab	27.1	b	28.4	ab
Mo	0.98	a	0.06	b	-		1.15	a	-	
Ni	1.04	a	0.92	a	0.69	ab	0.61	b	0.45	b
Pb	0.71	ab	0.93	a	0.16	c	0.85	a	0.23	c
Zn	201	ab	243	a	128	b	148	b	120	b

Improving phytoextraction

Many efforts have been made to improve phytoextraction of heavy metals, so that the effects of a great variety of treatments to biomass species are still being studied. Besides genetic improvements, plant responses to chelators, hormones and mycorrhizae have been investigated in order to assess plant tolerance and metal uptake, and the most appropriate doses and ways of application of these means.

Genetic engineering

Genetic engineering applied to crops aims at manipulating the plant's capacity to tolerate, accumulate and metabolise pollutants. Many genes involved in the acquisition, allocation and detoxification of metals have been identified and characterised from a variety of organisms, especially bacteria and yeasts (Ehrlich et al., 1997). Transgenic plants have been engineered to overproduce recombinant proteins playing possible roles in chelation, assimilation and membrane transport of metals. Enhanced tolerance and accumulation have been achieved through overproduction of metal chelating molecules such as citrate, phytochelators, metallothioneins, phytosiderophores and ferritin, or overexpression of metal transporter proteins.

Enhanced aluminium tolerance has been achieved by increasing organic acid synthase gene activity. Han et al. (2009) isolated a full-length OsCS1 gene encoding for citrate synthase, which is highly induced by Al toxicity in rice (*Oryza sativa* L.). Insertion of OsCS1 in several independent transgenic tobacco lines and its expression increased citrate efflux and conferred great tolerance to aluminium.

Overexpression of either gamma-glutamylcysteine synthetase or glutathione synthetase in transgenic *Brassica juncea* (L.) Czern. resulted in higher accumulation and tolerance of various metals such as Cd, Cr and As, considered alone or mixed together (Reisinger et al., 2008).

An attempt to improve tolerance to Cd, Zn and Ni was made by introducing a metallothionein gene in tobacco (Dorlhac de Borne et al., 1998; Pavlikova et al., 2004).

Macek et al. (2002) also showed that Cd accumulation significantly increased in tobacco plants bearing the transgene coding for the polyhistidine cluster combined with yeast metallothionein.

Another promising approach is the introduction of genes encoding for phytosiderophores. A first step in this direction was achieved by Higuchi et al. (1999), who isolated genes encoding for nicotianamine synthase, a key enzyme in the phytosiderophore biosynthetic pathway in barley and rice. The increase of iron acquisition mediated by phytosiderophores was found to provide an advantage under Cd stress in maize (Meda et al., 2007). Overproduction of ferritin through genetic modification also led to increased Fe uptake as well as Cd, Mn and Zn, but only at alkaline pH (Sappin-Didier et al., 2005). This was due to high pH Fe-deficiency, which stimulates metal uptake and translocation in shoots through an increase in root ferric reductase and H^+ -ATPase activities.

Chelating agents

The addition of natural and/or synthetic chelators has been extensively experimented in phytoextraction, in order to increase metal bioavailability, uptake and translocation of metals. This goal may be achieved by adding both inorganic and organic agents to the soil, although the latter appears to be more effective in increasing the solubility of metals (Schmidt, 2003; Quartacci et al., 2005; 2006).

Among various synthetic chelators, ethylene-diamine-tetraacetic acid (EDTA) has been tested more intensively (Blaylock et al., 1997; Huang et al., 1997; Grčman et al., 2001). This chelator has shown its ability to mobilise heavy metals from the soil-solid phase through stable metal-complexes available for uptake in pore water.

Metal-chelator complexes are taken up along an apoplastic pathway (Tanton and Crowdy, 1971) and they pass through the Casparian strip. The Casparian strip is not fully formed near the tip of roots, and it is also disrupted close to lateral branches (Tanton and Crowdy, 1971; Haynes, 1980). Through this pathway, components from the solution can enter the stele which houses the xylem without passing through a cell membrane (Haussling et al., 1988). In some species, water and solutes can also enter the xylem through passage cells, a small number of unsuberised endodermic cells (Clarkson, 1996).

In several cases, chelators have been found to increase metal translocation from roots to shoots, as revealed by Ensley et al. (1999) with EDTA and organic acids. In other experiments, improvements of metal mobility in soil and accumulation in roots only were observed (Lombi et al., 2001).

Application of EDTA has been found to improve the uptake of several metals, particularly Pb – as shown, for instance, in *Brassica juncea* (L.) Czern., with concentrations 1,000-10,000 times greater than those of controls (Blaylock et al., 1997). Due to the persistent nature of EDTA in the environment, the risk of metal leaching to ground- and surface-water may rise markedly (Chen et al., 2004; Sun et al., 2001), as well as toxicity to soil biota (Grčman et al., 2001).

As an alternative to EDTA, its structural isomer ethylene-ediamine-disuccinic acid (EDDS) has recently been proposed to enhance phytoextraction (Grčman et al., 2003; Luo et al., 2005). The [S,S]-isomer of EDDS is readily biodegradable in soil (Schowanek et al., 1997; Bucheli-Witschel and Egli, 2001) and its mineralisation, including metal chelates (Vandevivere et al., 2001) in sludge-amended soil has been estimated to be complete in 28 days, with a half-life of 2.5 days (Jaworska et al., 1999). The affinity of EDDS is mainly oriented towards Cu and secondly to Zn, and this chelator shows a solubilisation effect greater than that of EDTA at equimolar concentrations and pH 7 (Tandy et al., 2004). No toxic effects of EDDS or the Cu-EDDS complex have been found on soil biota (Kos and Leštan, 2004; Vandevivere et al., 2001). Tandy et al. (2006) found decreased concentrations in shoots of essential metals like Cu and Zn, and greater values for Pb in EDDS-assisted phytoextraction. These authors suggested that, in the presence of EDDS, the three pollutants are taken up by the non-selective apoplastic pathway as metal-EDDS complexes. In the absence of chelator, essential metal uptake is primarily selective along the symplastic pathway. This shows that synthetic chelating agents do not necessarily increase uptake of heavy metals, when soluble concentrations are equal in the presence and absence of chelates.

Some of the restrictions concerning chelate-assisted phytoextraction may be overcome by using easily biodegradable and poorly phytotoxic compounds, such as nitrilotriacetate (NTA) and low molecular weight organic acids, such as acetic, citric, oxalic, fumaric and succinic acids (Krishnamurti et al., 1997; Kulli et al., 1999; Kayser et

al., 2000; Chen et al., 2003; Wenger et al., 2003). The chelating effect of organic acids roughly follows the order citric > malic > acetic, indicating that the corresponding dosage should be increased from at least 2 mmol kg⁻¹ of soil (citric acid) to 15 mmol kg⁻¹ (tartaric acid) (Gao et al., 2003). Nitrilotriacetic acid combines high biodegradability with good chelating strength. In soils, it was found to degrade as fast as citric acid, and rapidly even in anaerobic conditions and at low temperatures (Ward, 1986). The chelating effect of nitrilotriacetic acid is weaker than that of EDTA, but greater compared with low molecular weight organic acids (Wenger et al., 2003; Quartacci et al., 2005; Ruley et al., 2006). Several studies have focused on the use of nitrilotriacetic acid as a ligand to assist metal phytoextraction (Wenger et al., 2003; Meers et al., 2004; Quartacci et al., 2005, 2006; Ruley et al., 2006), showing worse metal accumulation than with EDTA on *Brassica carinata* A. Braun and contamination by Cu, Pb and Zn (Quartacci et al., 2007).

Low molecular weight organic acids are natural compounds in plant root exudates, and can improve ion solubility and uptake thanks to their metal chelating properties, their indirect effects on microbial activity (stimulation), and the physical properties of the rhizosphere (Wu et al., 2003). The positive influence of organic acids has been shown in durum wheat, the Cd concentration of which is proportional to the abundance of these acids in the rhizosphere (Cieřliński et al., 1998). Citric acid also reduces the toxicity of Cd in radish, and stimulates its translocation from roots to shoots by converting the metal into more easily transported forms (Chen et al., 2003).

Environmental- and plant-safe use of chelators should consider modest dosages – from 3 mmol kg⁻¹ of soil for EDTA (Luo et al., 2006) to 5 mmol kg⁻¹ for EDDS (Quartacci et al., 2007; Bandiera et al., 2009a) – applied close to harvest (about 1 week before) and preferably near the root system.

Humic acids

Humic substances are widespread natural molecules that have a potential role in phytoremediation as alternatives to synthetic chelators. Humic acids represent the fraction of humic substances insoluble in water in acidic conditions, which become soluble and extractable at higher soil pH. Molecules of humic acids are characterised by acidic groups such as carboxyl and phenol OH functional groups (Hofrichter and Steinbüchel, 2001),

which play an important role in the transport, bioavailability and solubility of heavy metals (Lagier et al., 2000). At the same time, humic acids can contribute to environmental protection by reducing the physical mobility (diffusion, mass flow) of various metal species (e.g., Cu, Pb, Zn, Ni) in soil, and thus limiting the consequent risk of percolation into ground-water (Halim et al., 2003). Contradictory results have been obtained from the application of humic acids. Bianchi et al. (2008) found increased mobilisation of Cu and Zn, associated with both negligible phytotoxicity in *Paspalum vaginatum* Sw. and improved metal extraction. Better uptake of Mo in forage in a polluted valley in Austria (meadow rendzina) was observed after application of large quantities (10 g kg^{-1} w/w, i.e., 1%) of humic acids, as a consequence of the increased extractable fraction of Mo (+28%) (Neunhäuserer et al., 2001). Increased concentrations of humic acids in soil, from 0.125 to 1.25 mg kg^{-1} , improved the solubility of Hg in modified Hg-contaminated mine tailings, especially when thiosulphate salts (ammonium and sodium) were added to the medium (Moreno et al., 2005). The same authors showed that translocation of Hg to the shoot varied significantly among plant species. The high translocation rate observed in *Brassica juncea* (L.) Czern. in the presence of ammonium thiosulphate was suppressed when humic acids were also added to the nutrient solution, probably because of the retention of Hg-humic acid complexes at root level.

Improved phytoextraction efficiency for Cd was found by Evangelou et al. (2004) after the application of high dosages of humic acids, through increased Cd bioavailability in soil. The addition of humic acids at a very high dose (20 g kg^{-1} of soil) increased Cd concentration in the shoot from 30.9 to 39.9 mg kg^{-1} in *Nicotiana tabacum* L., but not at a dose of 10 g kg^{-1} – probably due to soil acidification or the formation of Cd-humic acid complexes easily absorbed by plants.

The hormone-like effect of humic substances has also recently been investigated in crops (Delfine et al., 2005) and forest species (Pizzeghello et al., 2000) – with contrasting results, depending on plant species, amount and method of application, soil pH, and interactions with soil microflora. In *Raphanus sativus* var. *oleiformis*, we found that low doses of humic acids to the soil (0.1 g kg^{-1}) had a growth-promoting effect, especially on roots, whereas high doses were phytotoxic (Bandiera et al., 2009b). At this low rate, humic

acids increased the amounts of various heavy metals taken up by the plant and their translocation to the shoot, with major benefits for Cu and Pb.

Auxins

The use of plant growth-promoting substances may potentially improve the phytoremediation of trace elements, since they positively increase both shoot biomass and root extension, which may allow greater acquisition of metals. Among hormones affecting plant growth, auxins play the major role, being involved in cell division, growth, maturation, organ differentiation, and several physiological processes (Trewavas, 2000). Auxins are also directly involved in cation uptake, having plasma membrane H^+ -ATPase as final target (Hager, 2003). H^+ -ATPase acidifies the apoplast, whereas the cytoplasm becomes alkalinised (Tode and Lüthen, 2001). Acidification of the apoplast weakens the cell wall, and the electrochemical gradient created across the plasma membrane leads to the opening of cation channels or activates membrane ion transport proteins, which results in influx of cations (Liphadzi et al., 2006).

Very few studies regarding the use of auxins in phytoremediation are available in the literature. Among these, Lopez et al. (2005) found increased Pb concentrations in roots (+40%) and leaves (28×) of *Medicago sativa* L. when the plants received 100 μ M of indoleacetic acid in addition to EDTA in the hydroponic solution. Liphadzi et al. (2006) also found improvements in metal uptake in *Helianthus annuus* L. when indolacetic acid was given to leaves and soil, regardless of concentrations of 3 or 6 $mg\ L^{-1}$ (i.e., 17-34 μ M). In non-EDTA-amended soil only, these authors observed increases in root growth which were associated with higher leaf Mn and Ni contents.

Plant growth can also be enhanced through bacterially produced phytohormones. For instance, Sheng and Xia (2006) found increased growth of both root and shoot and Cd accumulation in *Brassica napus* L. as a result of soil inoculation with Cd-resistant bacteria. In the experiment of Dimkpa et al. (2008), production of siderophores by various *Streptomyces* strains was found to promote auxin synthesis via chelation of metals such as Cd, Cu, Ni, having the potential to inhibit the synthesis of this hormone.

Mycorrhizae

Mycorrhizae have various positive effects on plants. Increases in nutrient uptake and production of hormones such as cytokinins and gibberellins may be exploited in phytoremediation. Mycorrhization occurs naturally in a very large number of species – more than 90% – with the exception of the *Brassicaceae* family (Arshad, 2007).

Mycorrhizal fungi are ubiquitous in soils, including disturbed or contaminated land, although pollution causes changes in the diversity and prosperity of their populations. High concentrations of heavy metals can delay, reduce or prevent mycorrhizal colonisation and spore germination. The presence of heavy metals in soils allows the selection of tolerant plant and fungi species, and the simultaneous presence of various metals can cause synergic or antagonist interactions, thus increasing or lowering the toxicity of one metal. Ectomycorrhizal fungi are sensitive to heavy metals, and interactions among metals may modify metal toxicity. For instance, reduced Cd toxicity was detected in the presence of Zn (Colpaert and Van Assche, 1992; Brunnert and Zadrazil, 1985; Hartley et al., 1997). The toxicity of Cd, Pb, Zn and Sb in combination was equal to that of Cd alone (Hartley et al., 1997).

Mycorrhizal fungi can improve phytoextraction by making heavy metals more available for plant absorption. Giasson et al. (2005) reported that, in the presence of a form of Zn unavailable for plants (ZnCO_3), the endomycorrhizal hyphae of *Glomus intraradices* can move Zn to water-soluble species. This phenomenon was even more evident with Cd. Zinc saturation was reached in *G. intraradices* colonised roots at around 400 mg kg^{-1} , regardless of initial ZnCO_3 concentrations; Cd saturation was not reached. Improved phytoextraction by mycorrhization may be achieved by several mechanisms: (i) better plant growth and biomass production; (ii) increased plant tolerance to metals; (iii) greater metal concentrations in plant tissues. Baum et al. (2006) showed improvements in Zn phytoextraction in willows after inoculation with *Paxillus involutus* (ecto-mycorrhizaea). The association between *Elsholtzia splendens* Nakai and various arbuscular mycorrhizal fungi (*Gigaspora* spp., *Scutellospora* spp, *Acaulospora* spp., *Glomus* spp.) increases both root and shoot growth and metal concentrations, and is more effective than symbiosis with *Glomus caledonium* alone in enhancing the removal of Cu, Zn, Pb and Cd (Wang et al., 2005). In the same way, sunflower inoculated with *Glomus intraradices* achieves greater Cr

removal, due to enhancement of both biomass production and Cr concentration (Davies et al., 2001).

Conclusions

Re-use of abandoned metal-polluted sites is often limited by the dangerous presence of several contaminants. These lands can gain in environmental value when cultivated for phytoremediation purposes, also allowing opportunities for managing the risks of pollutant dispersion in the long term. In this regard, field crops represent a reliable alternative to hyperaccumulators, although the process still lasts a long time.

In order to solve the main restrictive factors of phytoextraction with field crops, such as activation of unavailable heavy metals in soils and the low uptake and translocation of target metals, some strengthening measures should be taken. For this reason, phytoextraction is linked with genetic engineering and advanced agricultural practices. The use of chelators to enhance metal bio-availability, the application of phyto-hormones to increase plant growth, and mycorrhization may all facilitate the application of phytoextraction at a commercially large scale.

Indeed, the choice of species, one or several, and adjustment of cultivation technique need thorough study of the plant's potential and adaptability to a specific environment. Results are not easily predictable, and preliminary experiments in micro- and meso-cosms can only give some indications. The time-scales for remediation using phytoextraction are generally long, but there is current evidence that this process may be integrated with metal phytostabilisation, especially for tap-rooted species.

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Chapter II. Phytoremediation trials on metal- and arsenic-contaminated pyrite wastes (Torviscosa, Italy)²

Abstract

At a site in Udine, Italy, a 0.7 m layer of As, Co, Cu, Pb and Zn contaminated wastes derived from mineral roasting for sulphur extraction had been covered with an unpolluted 0.15 m layer of gravelly soil. This study investigates whether woody biomass phytoremediation is a realistic management option. Comparing ploughing and subsoiling (0.35 m depth), the growth of *Populus* and *Salix* and trace element uptake were investigated in both pot and field trials. Species differences were marginal and species selection was not critical. Impaired above-ground productivity and low translocation of trace elements showed that bioavailable contaminant stripping was not feasible. The most significant finding was of coarse and fine roots proliferation in surface layers that provided a significant sink for trace elements. We conclude that phytostabilisation and effective immobilisation of metals and As could be achieved at the site by soil amelioration combined with woody species establishment. Confidence to achieve a long-term and sustainable remediation requires a more complete quantification of root dynamics and a better understanding of rhizosphere processes.

Abbreviations: CRW: coarse root weight; DTPA: diethylene triamine pentaacetic acid; EC: root electrical capacitance; FRW: fine root weight; HM: heavy metals; RLD: volumetric root length density; SW: shoot weight; TRW: total root weight.

² From: Vamerali T., Bandiera M., Coletto L., Zanetti F., Dickinson N.M., Mosca G. (2009) Phytoremediation trials on metal- and arsenic-contaminated pyrite wastes (Torviscosa, Italy). *Environ. Pollut.* 157, 887-894.

Introduction

Phytoremediation is a low-cost technology that uses vascular plants for *in situ* environmental restoration and reclamation of contaminated soils, sludges and sediments (Salt et al., 1998). Clean-up of inorganics may be achieved by combining high productivity with elevated uptake and translocation of pollutants to the harvestable biomass (Chaney et al., 2007). Alternatively, phytostabilisation may be used to reduce the mobility, toxicity and dispersion of pollutants. Establishment of a vegetation cover can control erosion, create an aerobic environment in the rhizosphere, and provide organic matter in the soil, increasing soil aggregation and binding contaminants (Pulford and Watson, 2003; Robinson et al., 2006). Root tissues can also sequester heavy metals, especially those with lower mobility such as Pb and Cu (Del Rio Celestino et al., 2006; Marmiroli, 2005; Vazquez et al., 2006; Yoon et al., 2006), whilst root exudates can cause precipitation (Heim et al., 1999; Marmiroli et al., 2005; Yang et al., 2005) or mobilisation (Nigam et al., 2001). This may or may not be beneficial for phytoremediation.

The extended root system of trees is more suitable than that of herbaceous species to remediate deep polluted soil layers (Pulford and Dickinson 2005); preferential root proliferation in the polluted zones of the soil would be particularly beneficial for phytoextraction (Schwartz et al., 1999; Whiting et al., 2000). Two problems are that roots development may facilitate the contaminants entry to the food chain, and may provide macropores through which contaminants may leach (Roulier et al., 2008). Short-rotation coppice poplar (Laureysens et al., 2004a, 2004b) and willow (Dickinson and Pulford, 2005; Rosselli et al., 2003) may provide an efficient and cost-effective method to deal with soils polluted by heavy metals. For example, appreciable accumulations of Zn and Cd were found by Rosselli et al. (2003) in twigs of *Salix viminalis* (up to 294 mg kg⁻¹ for Zn, and 3.4 mg kg⁻¹ for Cd) and *Betula pendula*, but not in *Alnus*, *Fraxinus* or *Sorbus*, cultivated in sewage sludge in Switzerland. Specific clones of *Populus × euramericana* were also found suitable for phytoextraction in Zn-polluted substrates (Di Baccio et al., 2003). Phytostabilisation of heavy metals in poplar species and clones has also been reported by

various authors (French et al., 2006; Schnoor, 2000; Tognetti et al., 2004). Stabilisation of pollutants in the tissues of woody species may also be an opportunity for improved landscape value and simplified management (Paulson et al., 2003).

The aim of the present study was to investigate the phytoremediation potential of woody biomass species grown in arsenic- and metal-contaminated pyrite cinders discharged as a by-product of sulphur extraction in manufacturing of sulphuric acid. The objectives of this work were: (i) to study the establishment and growth of three poplar species (*Populus alba*, *P. nigra*, *P. tremula*) and *Salix alba*, (ii) to evaluate their potential for accumulation of heavy metals, and (iii) to explore appropriate means of soil cultivation and management with respect to strategies of phytoextraction and phytostabilisation.

Materials and methods

Site description

Metal-contaminated wastes derived from pyrite ore roasting (800 °C) for sulphur extraction were discharged (1938- 1970s) adjacent to the Caffaro chemical plant at Torviscosa (Udine, NE Italy, 45° 49' 23" N, 13° 16' 40" E, 3 m a.s.l.). The polluted area was included (Italian Ministerial Decree 468/2001) in the national clean-up priority list of polluted sites, entitled "Lagoon of Grado and Marano and adjacent rivers". Pyrite ore contains mainly pyrite (FeS_2), lesser amounts of chalcopyrite (CuFeS_2), sphalerite (ZnS), and magnetite (Fe_3O_4), and various trace elements. The cinder wastes extended for a depth of 0.7 m over a deep horizon of clay, thought to have prevented significant metal leaching, and had been covered with a less polluted gravelly soil 0.15-0.2 m in depth. The cinders were largely devoid of organic matter, poor nutritional status, pH 7.3 and relatively low

electrical conductivity (0.3 S m^{-1}). A fuller characterisation of the site with its resident herbaceous and woody flora was provided by Coletto et al. (2006).

Trials were conducted for two years (2004-2005) in pots, filled with pyrite cinders, and in 2005 directly at the polluted site. The pot cinders were collected in three positions within an on-site area of $50 \times 20 \text{ m}^2$ at depth intervals of 0.2-0.5 m. The total concentrations of metals were measured after homogenisation and before pot filling. Samples were air-dried and sieved (2 mm), and about 1 g DW of cinders was microwave acid-digested using EPA method 3051 (USEPA, 1995a). Before analysis by ICP-OES (SPECTRO CirOS Vision EOP, SPECTRO Analytical Instruments GmbH &

Co. KG, Kleve, D), the samples were filtered ($0.45 \mu\text{m}$ PTFE) and diluted to 25 mL with distilled water. DTPA-extractable fractions of Cu, Pb and Zn in the cinders were also analysed. Analysis was performed in duplicate on 50 g of homogenised dried cinders by extraction through 100-mL solution of DTPA (1.97 g L^{-1}), calcium chloride bihydrate (1.46 g L^{-1}), and triethanolamine (14.92 g L^{-1}), pH 7.3, shaken for 2 hours (60 cycles/min). ICP-OES analysis followed centrifugation (5 min, 5000 rpm).

Pot experiment

One-year-old bare rooted cuttings of *Populus alba* L. (white poplar), *P. nigra* L. (black poplar), *P. tremula* L. (European aspen) and *Salix alba* L. (white willow), obtained from plants naturally established at S. Giustina in Colle (Padova, NE Italy), were transplanted at the end of June 2004 into cylindrical opaque PVC pots ($\varnothing$ 0.25 m, ht 0.45 m). The pots were kept outside for two years at the experimental farm of the University of Padova at Legnaro and were weekly fully wetted with 1:10 diluted Hoagland solution from March to October. Three replicate plants grown in pyrite cinders were planted in a 0.15 m layer of unpolluted silty-loam soil (35.1% sand, 49.3% silt, 15.6% clay), taken from the experimental farm at Legnaro. Plants used as references were cultivated in pots filled with the same silty-loam soil and watered as before. Both cinders and soil were mixed with sand (1:1 w/w) to facilitate water drainage. The silty-loam soil had pH 8.2, organic matter 2.2%, N 0.1%, electrical conductivity 0.03 S m^{-1} , and contents of trace elements below Italian legal limits (Table 1).

Table 1. Total concentrations ($\pm$ S.D., $n=3$) of above-limit trace elements in cinders in comparison with Italian maximum permitted concentrations (Italian Ministerial Decree 471/1999, agricultural uses) and in reference uncontaminated silty-loam soil. DTPA-extractable fractions determined in duplicate.

Metal	Legal limit (mg kg ⁻¹)	Pyrite cinders			Reference soil
		Total concentration (mg kg ⁻¹)	[HM] : limit	DTPA-extractable (mg kg ⁻¹)	Total concentration (mg kg ⁻¹)
As	20	886 $\pm$ 16	44 : 1	n.d.	18 $\pm$ 2
Co	20	100 $\pm$ 8	5 : 1	n.d.	11 $\pm$ 0.6
Cu	120	1,735 $\pm$ 212	14 : 1	142.0	106 $\pm$ 5
Pb	100	493 $\pm$ 31	5 : 1	69.8	13 $\pm$ 2
Zn	150	2,404 $\pm$ 248	16 : 1	44.6	128 $\pm$ 37

The dynamics of root growth were monitored during the second year only (2005) by the non-destructive electrical capacitance (EC) method. Data were acquired weekly by a handheld digital electrical capacitance meter (ELC-131D, Escort Instruments Corporation, Taipei, Taiwan) in a circuit composed of a negatively-charged electrode (applied at the stem base), roots, soil nutrient solution, and a positively-charged electrode (earthed in the soil nearby). The method is based on the polarisation of biological membranes in the roots, and depends on the geometric and dielectric properties of the root system (Chloupek, 1972, 1977; Dalton, 1995). EC measurements are reported to be dependent on root surface area (i.e., length or weight) and root cell content (activity), as root cells exert considerable resistance to current passing through the root system.

After preliminary calibration, the selected parameters followed Preston et al. (2004), allowing the most stable EC values for repeated measurements (1-hour interval) within a 6-hour period:

a) positively-charged electrode: vertical stainless steel rod, 10 mm $\varnothing$, 550 mm long, 300 mm inserted into the potting soil (66% of pot height), and cable connection 200 mm above-ground level, earthed 50 mm away from the stem base;

b) negatively-charged electrode: fine stainless steel needle, orthogonally inserted into the stem at a height of 60 mm (1-mm penetration into the bark).

Capacitance values (pF) were recorded at 1 kHz frequency, 24 hours after watering (Hoagland solution) to reach approximately soil field capacity and to allow roots to be in contact with the soil solution.

Plant biomass was recorded at the beginning (woody stems only, 3 replicates) before transplanting, and end of the experiment (mid-October 2005, 465 days after transplant) by oven-drying (105°C, 36 hours). The final weights were measured for wood, leaves and roots separately. Roots were washed using floatation by means of a hydraulic centrifugation device, separately for the two soil horizons (depths of 0-0.15 and 0.15-0.45 m) as described by Oliveira et al. (2000), facilitating separation of soil particles through the addition of oxalic acid solution (2% w/v). Roots were collected in a 500- μ m mesh sieve. The weights of fine and coarse roots (about 2-mm threshold diameter) were also measured after manual separation and oven-drying.

Concentrations of As and trace metals were determined separately in samples of shoots (wood), leaves, and fine and coarse roots. About 0.4 g of dried biomass was added with 5 ml of nitric acid (70% v/v) and 1.5 ml hydrogen peroxide (30% v/v) and digested in microwave oven, following EPA method 3052 (USEPA, 1995b) alongside certified reference material. Samples were filtered (0.45 μ m PTFE), diluted to 25 mL with distilled water, and analysed by ICP-OES.

Field trial

In 2005, the same woody species as in the pot experiment were grown *in situ* at Torviscosa within a pilot phytoremediation system, which also included herbaceous species (not reported here). The site has a temperate climate (annual temperature 13.5 °C, precipitation 1200 mm). In 2005, the mean annual temperature and total precipitation were lower than mean data (12.7 °C, 1100 mm) and the growing season (May-September) was characterised by a mean temperature of 20.5 °C and 600 mm of precipitation. Cultivation was carried out after preliminary coarse sieving of the top carry-over soil, in order to remove the greater size fraction of the skeleton (> about 50 mm Ø). Following a split-plot experimental design, two different types of soil tillage (i.e., ploughing and subsoiling) were applied, both at 0.35 m depth. Essentially, ploughing involves a more thorough mixing of

soil than subsoiling which simply rips the surface. Despite preliminary sieving, the tilled layer still had about 60% of skeleton in both ploughed and subsoiled treatments. Tillage was intended to dilute the cinders with the less polluted top soil (ploughing) and to allow roots to encounter a better initial habitat (subsoiling). Because of excavation (for sieving) and shift of cinders during tillage, marked contamination of cinders was also verified in the top layer of the subsoiled treatment. Despite this, in fine soil ($< 2 \text{ mm } \varnothing$) the amount of cinders still remained different between treatments, i.e., higher in ploughing than in subsoiling (59% vs. 49%). The four species were also grown in a ploughed silty-loam soil of the experimental farm at Legnaro (reference uncontaminated, specifically for comparing plant growth).

Before transplanting, the soil was fertilised with 100 kg of N, P_2O_5 and K_2O per hectare, and then harrowed. Two-year-old bare cuttings (one-year-old for *P. tremula*) with naked roots were transplanted on mid-April in plots consisting of short rows of 6 plants each, with 3 replicates (a total of 72 trees for the 4 species). The distance between plants was 0.8 m, both within and between rows, to mimic as far as possible a short-rotation coppice system. Plots were irrigated when rainfall was insufficient to match the crop demand during summer. From June to the end of August, 30 mm of water were given 4 times, through a sprinkling system.

Volumetric root length density (RLD) and shoot dry weight were recorded at the end of the growing season, in comparison with the reference uncontaminated soil.

Root and shoot study in the field

Root systems were investigated at the beginning of October by auger sampling. One soil core (70 mm diameter, 0.3 and 0.7 m depths at Torviscosa and Legnaro, respectively) at two distances from the trunk (0.1 and 0.2 m) was taken for each plot. Root profiles were represented by subdividing each core into 0.1-m-deep sub-samples. Samples were stored at -18°C and root separation was achieved through flotation, as described above. Roots were stored at 4°C in ethanol solution (10% v/v). Subsequently, one-bit 375-DPI (i.e., $14.8 \text{ pixel mm}^{-1}$) TIFF-format images of roots were acquired by digital scanning on a flatbed scanner. Measurements of root length were performed by KS 300 image analysis software (Carl Zeiss Vision GmbH, München, Germany) following the procedure of Vamerali et al.

(2003). Briefly, the method allows the automatic discrimination of roots against extraneous objects (e.g., organic debris, small pebbles, seeds), based on their different shape. In this study, optimal discrimination of roots was obtained by adopting an Elongation Index value (i.e., $\text{perimeter}^2/\text{area}$) greater than 75. The length of roots was obtained by the function FbL (fibrelength), based on the perimeter (P) and area (A) of the digital objects, as follows:

$$FbL = \frac{P + \sqrt{P^2 - 16A}}{4}.$$

Root length was converted to volumetric root length density (RLD, cm cm^{-3}) by taking the volume of core sub-samples into account.

In mid-October, about six months after transplant, one plant per plot (shoots without leaves) was harvested for biomass determination (105 °C, 36 hours) and heavy metal analysis.

Statistical analysis

Statistical analysis on growth parameters, accumulation of heavy metals, and correlations (i.e., root parameters vs. trace element concentrations and removals in different plant organs; Table 3) were carried out by Statgraphics software (Manugistic Inc., Rockville, MD, USA). ANOVA and LSD tests were used to evaluate differences among means, following normality test of data.

Results

Metal content of cinders

Before mixing, the cinders had Fe (94.6%) and S (3.9%) as their main constituents, whilst As, Co, Cu, Pb and Zn considerably exceeded Italian guideline values (IGV) for agricultural uses (Italian Ministerial Decree 471/1999) (Table 1). Cadmium (0.46 mg kg^{-1}), Cr (2.5 mg kg^{-1}), Ni (3.1 mg kg^{-1}) and Mn (108 mg kg^{-1}) concentrations were not elevated. Subalkalinity (pH 7.3) was associated with low bioavailability of DTPA- extractable Zn (1.9%), but relatively high extractability of Cu (8.2%) and Pb (14.2%).

Pot experiment

All species showed appreciable but reduced growth after two years in the wastes (Fig. 1). Growth differences recorded between species in the uncontaminated soils were not reflected in their growth in the wastes (all organs). *P. alba* grew marginally better in the waste and *Salix* marginally worse. Growth of both coarse and fine roots was significantly reduced in the waste, except for the fine root fraction of *P. alba* (Fig. 2). Total root weight at harvest was correlated with measurements of root electrical capacitance (EC) during the second year, using all data combined from the cinder waste and reference soil. EC values from September-October, just before destructive sampling, provided the best correlation ($R^2 = 0.28$). The EC represented the total root mass better in the wastes than in the reference silty-loam soil, probably due to the higher electrical conductivity of the cinders. In order of root yields (*P. alba* > *P. nigra* > *S. alba* > *P. tremula*) the pattern was the same. In the waste, EC values were generally lower than those of the reference uncontaminated soil, apart from *P. alba* and *P. tremula* during most of the summer (Fig. 3).

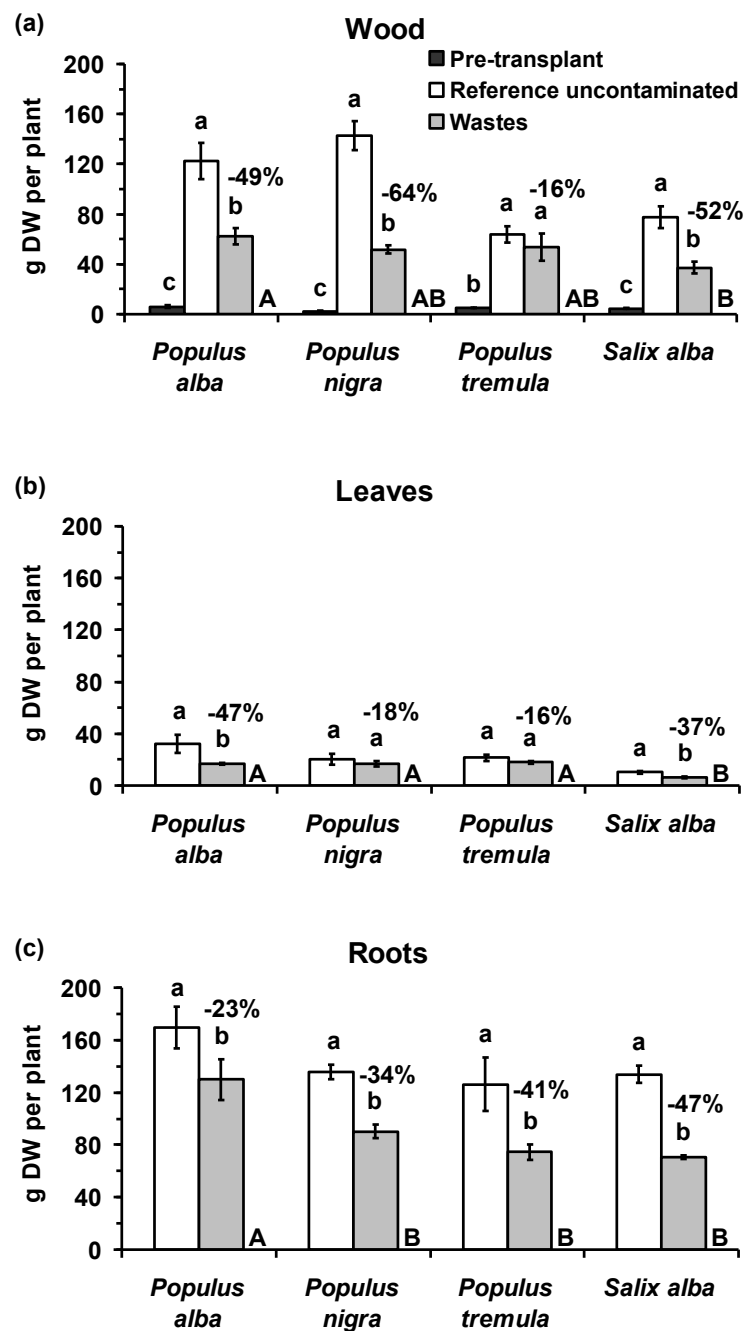


Figure 1. Pot experiment: dry weights of plant organs at harvest, after two years of cultivation ($\pm$ S.E., $n=3$). Capital letters: statistically significant differences among species grown in wastes; small letters: differences between treatments within same species (LSD test; $P \leq 0.05$). Percentages refer to variations with respect to reference uncontaminated.

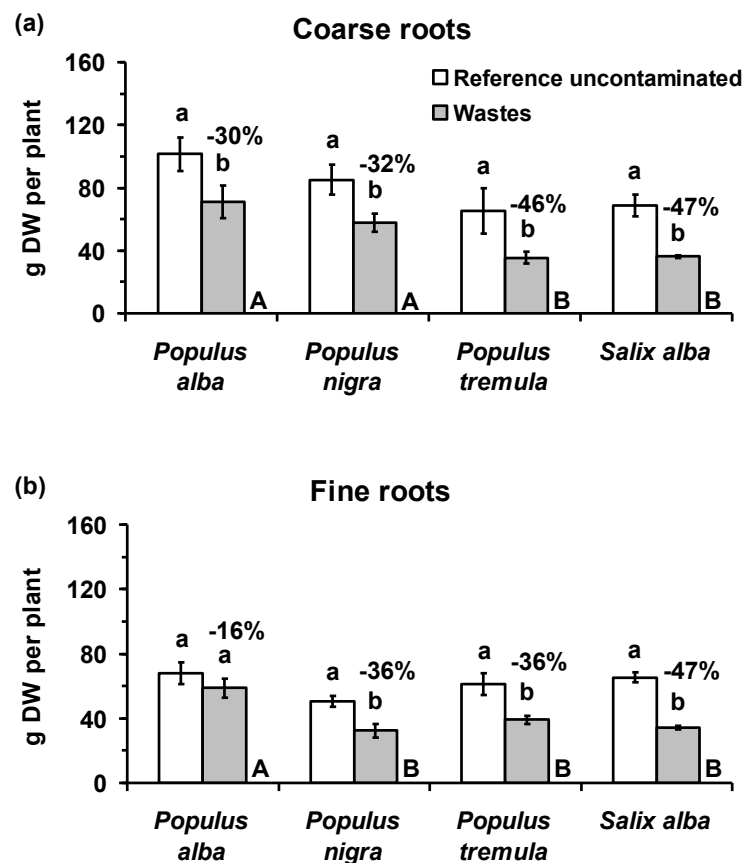


Figure 2. Pot experiment: dry weights of coarse and fine roots ($\pm$ S.E., $n=3$). Capital letters: statistically significant differences among species grown in wastes; small letters: differences between treatments within same species (LSD test; $P \leq 0.05$). Percentages refer to variations with respect to reference uncontaminated.

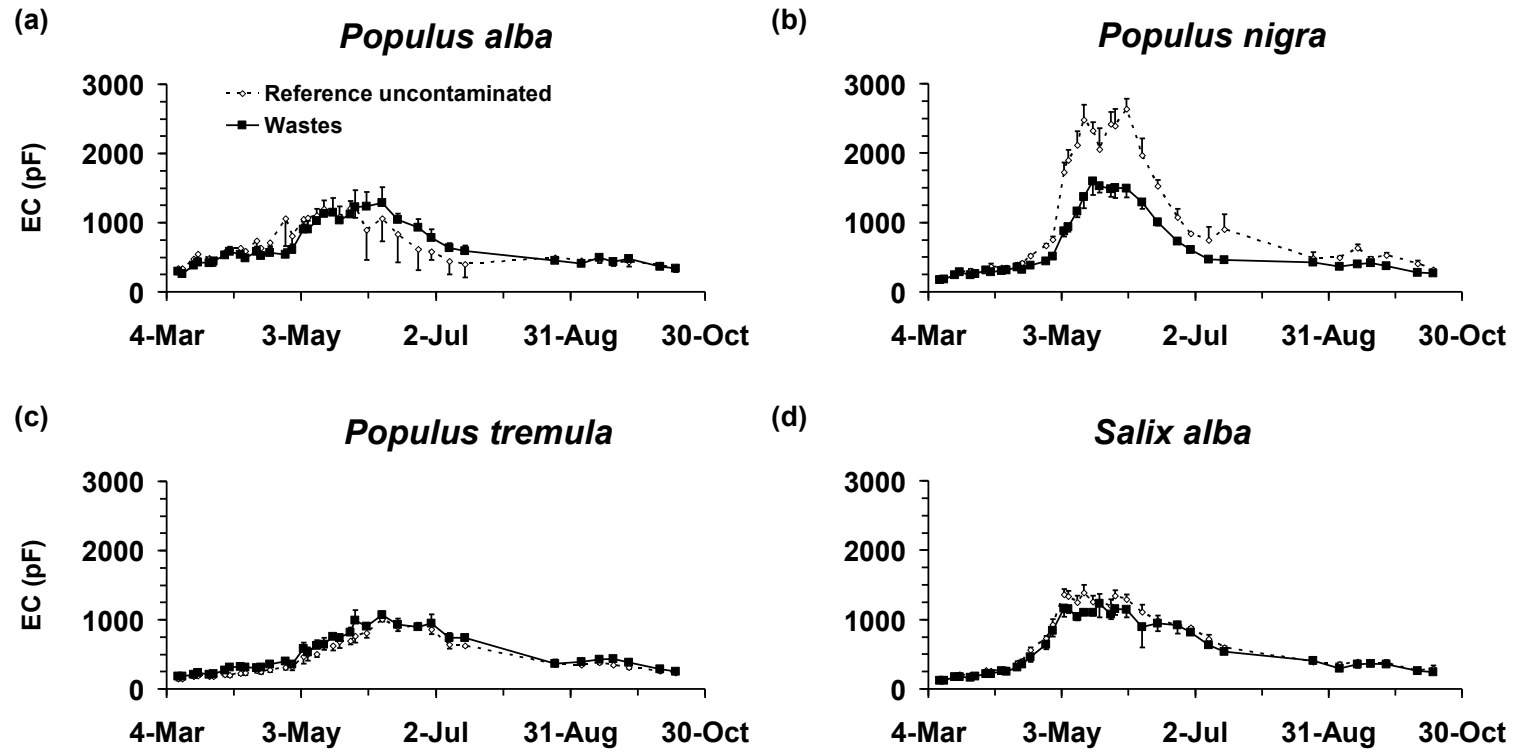


Figure 3. Pot experiment: dynamics of root electrical capacitance (EC) of various species ($\pm$ S.E., $n=3$) during 2005 growing season (2nd year).

Table 2. Pot experiment: concentrations of trace elements (mg kg⁻¹) ±s.e. in plants (n=3) grown in cinders. For same pollutant, capital letters indicate statistically significant differences among plant organs, small letters differences among species for the same organ (LSD test; P≤0.05). Missing values: below detection limit of 1.4 mg As kg⁻¹ and 0.21 mg Co kg⁻¹.

Organ	Species	[As]		[Co]		[Cu]		[Pb]		[Zn]	
Wood	<i>Populus alba</i>	-		-		6.65±0.62	ab	11.7±1.88	a	56.7±6.58	ab
	<i>Populus nigra</i>	-		-		5.64±0.17	b	4.45±0.82	a	59.2±7.13	ab
	<i>Populus tremula</i>	-		-		8.00±0.84	ab	11.2±4.06	a	47.7±4.46	b
	<i>Salix alba</i>	-		-		10.4±2.21	a	6.48±2.01	a	77.9±13.7	a
	MEAN	-		-		7.70±0.75	B	8.50±1.41	B	60.4±4.98	C
Leaves	<i>Populus alba</i>	2.42±0.39	b	0.70±0.10	a	6.83±0.27	b	2.98±0.25	ab	287±23.8	b
	<i>Populus nigra</i>	1.96±0.28	b	0.52±0.05	a	5.86±0.11	c	2.63±0.52	ab	318±46.4	b
	<i>Populus tremula</i>	2.27±0.63	b	0.65±0.06	a	6.16±0.33	bc	2.10±0.11	b	223±28.9	b
	<i>Salix alba</i>	5.05±0.54	a	0.75±0.07	a	7.69±0.17	a	3.24±0.25	a	510±41.5	a
	MEAN	3.00±0.46	B	0.70±0.04	B	6.60±0.24	B	2.70±0.19	B	335±35.7	A
Coarse roots	<i>Populus alba</i>	20.2±6.45	b	0.35-	a	133±17.1	b	6.14±1.27	b	37.2±1.34	b
	<i>Populus nigra</i>	70.4±15.9	a	0.72±0.15	a	281±57.7	a	17.9±3.30	a	52.9±4.41	a
	<i>Populus tremula</i>	18.3±12.2	b	-		107±59.9	b	4.12±2.26	b	24.7±1.74	c
	<i>Salix alba</i>	10.5±4.83	b	0.31±0.04	a	86.9±24.0	b	4.32±1.02	b	52.6±3.72	a
	MEAN	30.9±8.94	B	0.50±0.11	B	152±29.7	B	8.10±1.95	B	41.9±9.00	C
Fine roots	<i>Populus alba</i>	77.9±37.8	a	2.49±0.51	a	853±209	a	34.0±9.31	a	133±15.6	a
	<i>Populus nigra</i>	145±77.1	a	2.63±0.34	a	720±240	a	43.5±14.9	a	136±22.7	a
	<i>Populus tremula</i>	62.0±13.1	a	2.84±0.66	a	1,160±286	a	44.7±8.28	a	111±14.1	a
	<i>Salix alba</i>	59.2±14.8	a	2.06±0.07	a	680±102	a	28.8±2.70	a	155±18.5	a
	MEAN	85.9±21.5	A	2.50±0.21	A	853±110	A	37.8±4.64	A	134±3.78	B

Trace element concentrations were much higher in roots than in above-ground tissues, with particularly high concentrations in fine roots (Table 2). Differences between species were most pronounced in the older coarse roots, for which the highest accumulations were measured in *P. nigra* (all pollutants, except Co) and *S. alba* (Zn). Of course contamination of roots with soil is likely to have introduced some errors. In wood, highest concentrations of Cu (10 mg kg^{-1}) and Zn (78 mg kg^{-1}) were in *S. alba*, but there were relatively modest variations of these metals between species (C.V.: Cu 27%, Zn 21%). *Salix alba* foliage contained highest concentrations of As (5 mg kg^{-1}), Cu (7.7 mg kg^{-1}), Pb (3.2 mg kg^{-1}) and Zn (510 mg kg^{-1}); leaf Zn concentration exceeded those of wood by almost 6 times. In terms of mass balance, the overall removal of trace elements (whole plant, 5 trace elements combined) was only significantly higher in *P. alba* than in *S. alba* (Fig. 4); *Populus alba* (88 mg per plant), *P. nigra* and *P. tremula* (both 66 mg), *S. alba* (44 mg). Small differences between species were found in metal uptake to wood and foliage. Accumulating in roots was much higher than in above-ground biomass and the root system contained the major fraction of pollutants removed from the waste (varying from 84% in *S. alba* to 89% in *P. alba*). Zinc accounted for the largest proportion of trace elements transferred to foliage (96% on average) and wood (78% on average).

The biomass of both root fractions was positively correlated with Zn uptake (Table 3), and the coarse root fraction biomass was positively correlated with the concentrations of Cu, Pb and Zn in leaves. The biomass of deep fine roots was also positively correlated with Co, Cu and Pb in leaves, but often negatively with root concentrations of most trace elements (except Zn).

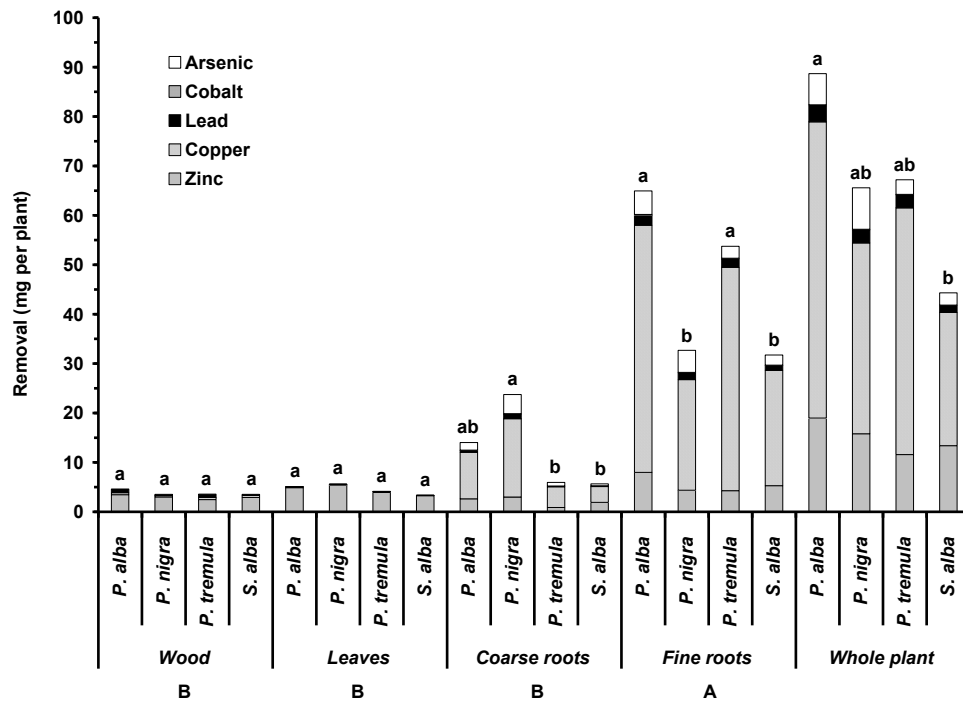


Figure 4. Pot experiment: overall removal of As and trace metals in various organs and in whole plants of species grown in wastes (n=3). Small letters: differences among species within same organ (LSD test; $P \leq 0.05$). Capital letters: differences among organs (main effect).

Table 3. Pot experiment: correlations between root parameters and concentrations of trace metals (n=12, i.e., 4 species $\times$ 3 replicates). Coefficient of correlation (r) and significance are listed for each correlation. Variables are: TRW⁽¹⁾ = total root dry weight; CRW= coarse root dry weight; FRW= fine root dry weight; deep⁽²⁾ = fraction of root dry weight in 0.15-0.45-m depth interval (waste layer); ECint⁽³⁾ = seasonal root electrical capacitance (integral); SW⁽⁴⁾ = shoot dry weight (wood+leaves); Σ [HM] μ mol⁽⁵⁾ = summation of concentrations (μ Moles) of As, Co, Cu, Pb and Zn. Significant differences only are shown [* P $\leq$ 0.05; ** P $\leq$ 0.01; *** P $\leq$ 0.001].

Concentration	Organ	TRW ⁽¹⁾	CRW (deep) ⁽²⁾	FRW (deep) ⁽²⁾	ECint ⁽³⁾ /SW ⁽⁴⁾
As	Leaves	+0.75 **			+0.84 ***
	Coarse roots			-0.49 *	
	Fine roots			-0.40 *	
Co	Leaves			+0.62 **	
	Fine roots			-0.34 *	
Cu	Wood	+0.55 *		+0.38 *	
	Leaves	+0.70 *	+0.52 *	+0.52 **	
	Coarse roots			-0.52 **	
	Fine roots			-0.58 **	+0.41 *
Pb	Leaves	+0.51 *	+0.50 *	+0.59 **	
	Coarse roots			-0.45 *	
	Fine roots			-0.58 **	
Zn	Wood	+0.70 *			+0.51 *
	Leaves	+0.87 ***	+0.61 *		+0.84 ***
	Coarse roots	+0.79 **	+0.75 **		+0.67 ***
	Fine roots	+0.57 *	+0.50 *		+0.61 **
Σ [HM] μ mol ⁽⁵⁾	Wood	+0.70 *			+0.47 *
	Leaves	+0.87 ***	+0.61 *		+0.84 ***
	Coarse roots			-0.47 *	+0.37 *
	Fine roots			-0.54 **	+0.44 *

In situ experiment

In the field experiment, considerable growth reduction was evident both above- and below-ground, in both the ploughed and subsoiled treatments. Although the plants had been fertilised, leaf chlorosis was common with lower leaf SPAD values (chlorophyll content) than in reference plants in mid-June. Chlorophyll reduction occurred irrespective of soil management (data not shown). At the end of both pot and field investigations, *P. alba* had highest growth, and *P. nigra* showed most growth inhibition compared with the reference soil. There was no significant difference between ploughing and subsoiling (Fig. 5).

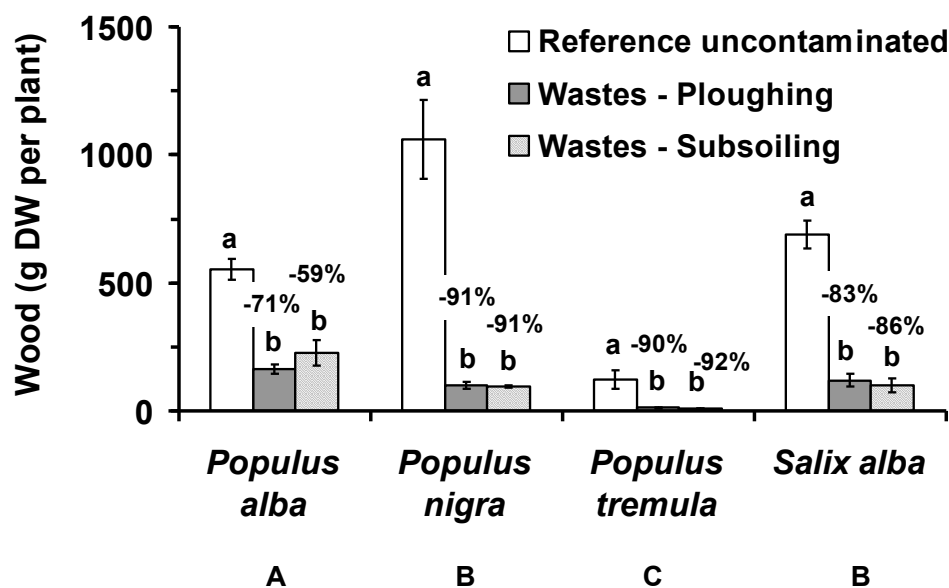


Figure 5. Field trial: woody biomass of species in different soil managements (n=3). Vertical bars: $\pm$ standard error. Capital letters: statistically significant differences among species grown in wastes; small letters: differences among treatments within the same species (LSD test; $P \leq 0.05$). Percentages refer to variations with respect to ploughed reference uncontaminated soil.

As, Co and Pb had not accumulated in twigs, and Cu concentration were low (Table 4). However, Zn was higher in the subsoiled plots than in ploughed plots (94 vs. 75 mg kg⁻¹), with higher uptake than in pots (60 mg kg⁻¹). Lowest Zn was in the best-growing

species, *P. alba*, and highest in *P. tremula*. Summation of concentrations (μMoles) of 6 trace elements in woody tissue (Cu, Zn, Ni, Cd, Cr, Mn) showed most uptake in *P. tremula*, with no significantly higher concentrations in the subsoiling plots compared to ploughed.

Root depth penetrations into the cinder waste was limited for all species, compared to growth in the reference soil (Fig. 6), although roots were much more concentrated in the surface 0.3 m. Root length did not differ between sampling positions, 0.1 and 0.2 m from the trunk base, allowing these data to be used as replicates. The average RLD of the surface rooted horizon was 6.3 and 4.5 cm cm^{-3} in subsoiled and ploughed plots (main effect), respectively, without significant interactions between soil tillage and plant species. No differences in root diameter were found between treatments or between species. Total root length was weakly but significantly correlated with the combined concentrations of the 6 trace metals in wood ($R^2=0.14$).

Table 4. Field trial: concentrations of trace metals (mg kg^{-1}) ($n=3$, $\pm\text{S.E.}$) in twigs of various species in two soil managements. For same metal, capital letters indicate statistically significant differences among treatments, small letters differences among species within same tillage (LSD test; $P\leq 0.05$).

Treatment	Species	Cu		Zn	
Ploughing	<i>Populus alba</i>	4.95 $\pm$ 0.23	bc	62.1 $\pm$ 0.95	b
	<i>Populus nigra</i>	4.01 $\pm$ 0.50	c	76.8 $\pm$ 10.1	b
	<i>Populus tremula</i>	9.11 $\pm$ 1.42	a	106 $\pm$ 8.64	a
	<i>Salix alba</i>	7.61 $\pm$ 1.17	ab	56.7 $\pm$ 8.47	b
	MEAN	6.42 $\pm$ 0.74	A	75.3 $\pm$ 6.64	B
	C.V. (%)	36.6		29.1	
Subsoiling	<i>Populus alba</i>	4.41 $\pm$ 0.15	b	65.4 $\pm$ 8.19	b
	<i>Populus nigra</i>	4.31 $\pm$ 0.14	b	103 $\pm$ 7.59	a
	<i>Populus tremula</i>	6.59 $\pm$ 0.15	a	116 $\pm$ 11.6	a
	<i>Salix alba</i>	7.50 $\pm$ 0.61	a	93.8 $\pm$ 12.4	ab
	MEAN	5.71 $\pm$ 0.44	A	94.4 $\pm$ 7.06	A
	C.V. (%)	27.9		22.6	

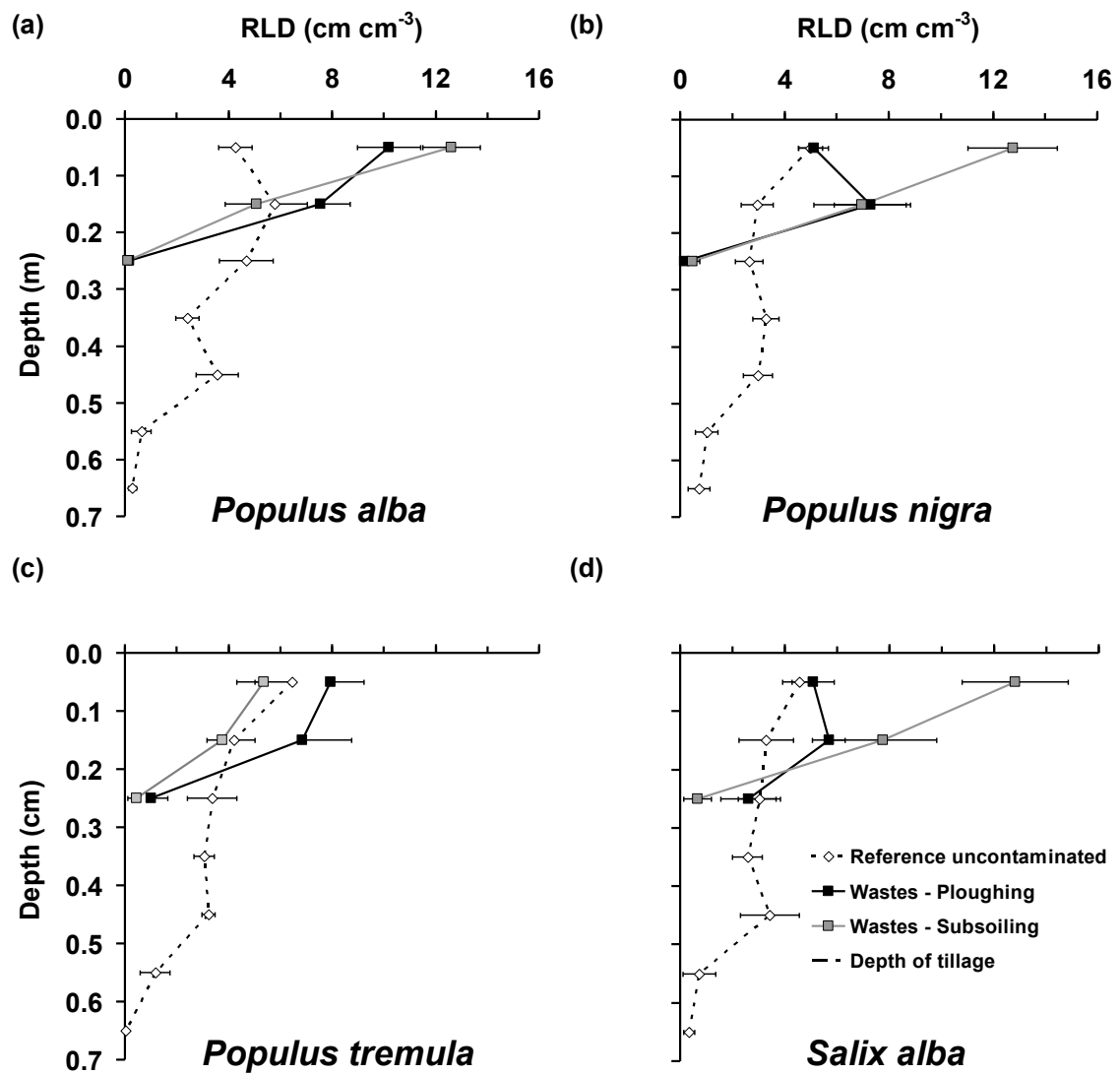


Figure 6. Field trial: patterns of root length density (RLD) of various species under varying managements of wastes, in comparison with reference uncontaminated soil (ploughed) ($n=3$). Horizontal bars: $\pm$ standard error.

Discussion

The pyrite cinder wastes provided an inhospitable substrate for plant growth; cultivation of *Populus* and *Salix* was difficult, as similarly found for herbaceous species (Fellet et al., 2007). Extreme physical properties of this substrate combined with high Fe, S and multiple trace element contamination strongly limit plant growth, regardless of species. In view of the high contents of As and Pb, in particular, there is undoubtedly a potential exposure risk to human health, groundwater and other receptors through windblow or leachate transfer. As exceeded the Italian limit much more than the other pollutants, and Pb had the highest DTPA-extractable fraction that may enhance its environmental risk. Simply covering the waste with a thin layer of imported soil had formerly proven to support an inadequate vegetation cover to stabilise the waste.

Mixing the cinder wastes with sand and covering with a layer of topsoil / sand in pot experiments provided a useful insight into the feasibility of establishing woody shrubs. Mineral fertilisers were probably essential to improve soil fertility for plant growth; earlier work in the same substrate showed substantial productivity gain in *Sorghum bicolor* and *Helianthus annuus* (14 and 9 times, respectively) with mineral and cow manure fertilisation (Marchiol et al., 2007). Nevertheless, biomass yield in the pot experiment was far below that in the silty-loam soil used as a reference, although root growth was less impeded than shoot growth. Biomass of the fine root fraction in *P. alba*, the best performing species in the waste treatment (in both pot and field experiment), was no different to that in the reference uncontaminated soil.

Trace element concentrations were also particularly high in the fine roots, although there was actually a negative correlation between fine root biomass and most trace element concentrations in fine roots. In general, fine roots had greater affinity for trace metals than coarse roots and shoots, particularly for Cu and As, whereas Zn was preferentially concentrated in leaves. There was usually a positive correlation between the amount of roots and uptake of trace elements to wood and foliage. Development of the root system may hold the key to understanding the possibilities and options for phytomanagement of

the wastes. Fine roots have a fast turnover of up to 90% within the same year in both coniferous and deciduous species, including poplar (Majdi et al., 2007; Ruess et al., 1996). Much remains to be understood about the mobility (or immobilisation) of metals in the rhizosphere and the role of coarse and fine roots (Dickinson 1982; Punz and Sieghardt, 1993; Turner and Dickinson, 1993; Whiting et al., 2003).

The provision of a non-destructive method for determination of root growth and metal uptake in woody plants would be particularly useful. The electrical capacitance method appears to allow non-destructive real-time analysis although, in the present study, the correlation between root EC and total root mass was much lower than that found by Preston et al. (2004) for one-year-old hybrid poplar saplings ($R^2 = 0.63$ vs. 0.89). Nonetheless, EC was closely related to As in foliage, Cu in fine roots, Zn in all plant parts and to the overall concentration of the 5 pollutants in the plants (Table 3). Integration of EC during the second year of the experiment showed a significant relationship with the total outputs of the 5 pollutants in wood tissues ($R^2=0.50$, $P\leq 0.001$) but not in whole plants. Contrary to the earlier studies (Preston et al., 2004) the EC technique proved to be of limited value in the present study.

There were small differences between species in terms of metal uptake, largely because of a lack of significantly transferred to above-ground tissues, as similar found elsewhere (e.g., Marchiol et al., 2007). In the case of As in foliage, *S. alba* had the maximum leaf concentration of 5 mg kg^{-1} , were probably within normal concentrations in terrestrial plants (Allen, 1989; Tu et al., 2002). *Salix* is known to preferentially accumulate Zn (as well as Cd) but in the present study Zn were 3-4 times lower than reported previously (Keller et al., 2003; Rosselli et al., 2003) and Cu concentrations in wood (range: $7.5\div 10.4 \text{ mg kg}^{-1}$) were similar. In coarse roots, the maximum metal stock was found in *P. nigra* – followed by *P. alba* – with values 4 times greater than in *P. tremula* and *S. alba* (24 vs. 6 mg per plant) (Fig. 4). It is interesting to note that the elevated elements in the pot experiment (As, Co, Cu, Pb, Zn) differed to those in the field experiment (Cd, Cr, Cu, Mn, Ni, Zn).

In the field trial, the ploughed treatment soils had a higher proportion of cinders than the subsoiling treatment soils that were just ripped. The results showed that greater root growth could be achieved in this way, rather than by ploughing and mixing the soil with the

waste. Creating almost normal conditions in the top soil allows establishment and appreciable growth, albeit with highly restricted rooting into the deeper polluted layer. All species rooted only shallowly (maximum 0.3 m) in the two treatments, highlighting the need for appropriate agricultural practices to enhance root growth.

The higher proportion of cinders in the ploughing treatment was reflected in tendency towards higher metal uptake. It is well established that plant roots forage for better quality localised soil zones with less growth constraints (Turner and Dickinson, 1993; Millis et al., 2004; French et al., 2006), but the toxic impact of the cinders at Torviscosa is obviously severe. Other soil treatments and practices could also offer improvements to be investigated (Dickinson, 2002). Promising increases in root length (+46%) have recently been found in *Raphanus sativus* L. var. *oleiformis* by soil application of small amounts (0.1 mg kg⁻¹) of humic acids (Bandiera et al., 2007).

Total root length was significantly correlated with the uptake of the 6 trace metals in wood ($R^2=0.14$), but overall uptake was modest and unlikely to be a significant risk to the wider environment (see Meeres et al., 2007; Vandecasteele et al., 2005). Longer roots would be expected to lead to more metal allocation to the wood, but actually root length was more associated with allocation of Zn to the leaves. It was considered that one possible approach to phytomanagement was Bioavailable Contaminant Stripping (BCS): using phytoextraction to remove only the most labile metal pools (Hamon and McLaughlin, 1999). However, high above-ground harvestable yields and trace elements uptake are essential for this to be feasible (Chaney et al., 2007; Ernst, 2005; McGrath and Zhao, 2003; Van Nevel et al., 2007), and this is unlikely to be achievable using woody biomass species at Torviscosa. Phytostabilisation (Dickinson et al., 2009) is a more feasible technique at Torviscosa, and there is need to immobilise the high levels of readily mobile Pb and As. Combined with the existing high pH of the substrate, an additional accumulation of organic matter would probably resolve issues with Pb. Reduced As mobility would be more dependent on Fe-oxides in soil lattices, maintenance of low phosphate conditions and lack of waterlogged conditions (Hartley et al., 2004). It is recognised that we require more knowledge of long-term trace element mobility in remediated wastes. The present work has pointed to the likely role of plant roots and especially fine root fractions in this dynamic.

Conclusions

Phytoextraction of elevated metals or As from Torviscosa cinder wastes using woody biomass species is not feasible, largely due to low productivity and low levels of translocation of trace elements from roots and despite an apparent high mobility of Pb. Zn, a largely non-zootoxic element, is the only possible exception. The present work has shown that establishment of *Populus* and *Salix* species at the site is achievable through ripping of the surface, minimal tillage, some mixing of the wastes with imported soil, irrigation and fertilisers.

Species differences were marginal and species selection was not critical, although mixed-species and mixed-clonal woody biomass planting is generally advisable for short-rotation coppice (Pulford and Dickinson 2005). There is also much scope for the addition of other soil amendments such as organic wastes, as demonstrated elsewhere (Clemente et al., 2006, 2008). These perennial plants do play an important landscape and ecological role, and it is likely that a vegetation cover would reduce windblow of soil and re-entrainment of particulate pollutants that may impact on human health or other receptors. Furthermore, uptake of metals and As to above-ground tissues is marginal and there is little risk of food web contamination via herbivores. In these contexts some degree of phytostabilisation can be readily achieved at the site, but questions remain about whether this would provide a long-term and sustainable remediation. Potentially, the elevated concentrations of Pb, As and other elements could be leached from the remediated wastes towards groundwater or other receptors, and these fluxes could also be influenced by soil amendments, changes in the rhizosphere or both.

Phytostabilisation and reduction of the mobility of contaminants of the polluted pyrite wastes at Torviscosa appears a much more realistic management option than phytoextraction. The present work has shown that roots *per se* are sinks for labile metals. Immobilisation of trace elements in both coarse and fine roots may reduce leaching, particularly of Cu and Zn but also As and Pb. Quantification of root turnover and a better

understanding of rhizosphere processes are required to establish the exact contribution of fine roots in long-term metal immobilisation.

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Chapter III. *In situ* phytoremediation of metal-polluted pyrite wastes with field crops under differing soil managements

Abstract

An *in-situ* phytoremediation trial was carried out at Torviscosa (Udine, NE Italy) in pyrite wastes, severely contaminated by As, Cd, Co, Cu, Pb and Zn, which had been discharged in the past and later covered with an unpolluted 0.15-m thick layer of gravelly soil. Sunflower (*Helianthus annuus* L.), alfalfa (*Medicago sativa* L.), fodder radish (*Raphanus sativus* L. var. *oleiformis* Pers.) and Italian ryegrass (*Lolium multiflorum* Lam.) were cultivated under two soil managements, ploughing vs. subsoiling (0.3 m depth) comparing plant growth and metal uptake.

Subsoiling caused undesired contamination by pyrite cinders in top soil, but mixing wastes with the unpolluted layer (ploughing) led to higher total and bioavailable metal concentrations. The choice of tillage system was not critical for mass balance of phytoextraction, but the effect on above-ground productivity and its metal concentrations diverged.

Raphanus sativus and *H. annuus* were the highest biomass-yielding species, but *Raphanus sativus* only reached higher metal offtake. Removals were generally poor, and followed the order fodder radish > Italian ryegrass > sunflower > alfalfa, i.e., 33, 21, 17 and 9 mg m⁻² per year, respectively, with zinc and copper as main harvested contaminants. Root growth was a key trait in phytoextraction, as shown by the highest root length density and shoot metal concentrations in *L. multiflorum*.

We conclude that phytomanagement of pyrite wastes with crop species is a feasible option, although agronomic practices should be managed appropriately to improve plant growth, root deepening and metal translocation. Confidence to achieve better metal

removals requires substantial soil amelioration and appropriate adjustment of the cultivation method applied.

Abbreviations: RLD: root length density; TF: translocation factor.

Introduction

After the discovery of hyperaccumulator plants (Raskin et al., 1994; Salt et al., 1995), the use of plant-based technologies to remediate metal-contaminated soils has received increased attention. The method consists of removing toxic metals through the harvestable biomass, after appreciable translocation from roots to shoots has occurred.

Although promising, phytoextraction has some limitations, due to poor plant establishment, limited metal availability in soils, difficulties in root uptake, symplastic mobility and xylem loading, as well as the great energy costs required for detoxification and storage within shoots (Meagher, 2000; Clemens et al., 2002).

Besides the use of hyperaccumulators, biomass crop species may represent a realistic alternative for removing trace metals (Vamerali et al., 2009b). Biomass species can absorb a wide range of metals at generally low concentrations, a feature compensated by higher biomass productivity. Introduction of crop species in phytoremediation is facilitated by the availability of seeds on the market, but their adaptability and cultivation method should be studied in each specific polluted site. Various treatments have been tested to improve phytoextraction by these species. For instance, soil amendment with low toxic organic chelators such as NTA and EDDS was found to increase metal uptake in *Brassica carinata* to a significant extent (Quartacci et al., 2007). Hormonal applications may result in higher plant growth and metal accumulation, as observed in alfalfa and sunflower (Lopez et al., 2005; Liphadzi et al., 2006), and mycorrhization can improve metal acquisition in maize

(Shen et al., 2006; Wang et al., 2007). Species selection and corrective treatments are often investigated in the greenhouse or laboratory, making transferral of results to the open field problematic, and currently only very few *in situ* experiments are being carried out.

Among metal-polluted media, major concern focuses on industrial wastes or sediments, the unusual compositions of which may further limit plant establishment and growth. A particular contamination case is represented by pyrite ore and its wastes remaining after sulphur extraction, mainly composed of fine particles of pyrite (FeS_2) and other minerals (Vidal et al., 1999). Oxidation of metal sulphides from pyrite residues releases soluble metals and increases soil acidity (Clemente et al., 2006), with the risk of metal leaching. Phytomanagement of pyrite wastes is an interesting and inexpensive option to reduce wind erosion and metal percolation, but little information is available in the literature on this issue. Pot cultivation of *Glicine max*, *Sorghum bicolor*, *Zea mays* and *Helianthus annuus* was reliable at various rates of pyrite dilution (Fellet et al., 2007). Instead, in the open field, initial evaluation of the phytoremediation potential of herbaceous crops (Clemente et al., 2005, 2006; Marchiol et al., 2007) and woody species (Vamerali et al., 2009a) has highlighted great reduction in plant growth. The above authors highlighted that species choice and soil amendment with fertilizers and lime are important in improving plant growth and metal removal.

In pyrite as well as in many metal-contaminated wastes, plant growth is limited by several environmental variables, such as low pH, salinity, insufficient aeration or low water and nutrient availability (Robinson et al., 2006). In these conditions, extensive root colonisation is an essential key to plant adaptation and uptake of metals, as has been shown for nutrients. Release of root exudates and rhizosphere acidification contribute to the absorption of metals (Nigam et al., 2000; Lasat, 2002) and improve metal tolerance (Pellet et al., 1995; Larsen et al., 1998).

Root behaviour is known in the hyperaccumulators *Thlaspi caerulescens*, which colonises predominantly Zn-polluted soil areas (Schwartz, 1999; Saison et al., 2004) and *Minuartia verna*, mainly growing spontaneously in Zn and Cd 'hotspots' (Cleal, 1994). Most biomass species appear to be very sensitive to metals below ground, as roots can be seriously damaged and reduced in growth (Ubi and Osodeke, 2007; Rascio et al., 2008). For example, disruption of the root cuticle, reduced root hair proliferation, and severe

deformation of root structure are caused by copper in *Chloris gayana* (Sheldon and Menzies, 2005).

Within this framework, the present study aimed at examining in the field the growth potential of various crop species in metal-polluted pyrite wastes at both above- and below-ground level. In particular, root length density was analysed in relation to metal accumulation in plants. As the wastes in question had been covered in the past by a thin layer of unpolluted soil, the phytoremediation potential was evaluated in two tillage systems, ploughing and subsoiling, in order to identify the best soil management.

Materials and methods

Description of polluted site

The study area covers 2,000 m² at Torviscosa (Udine, Italy, 45° 49' 23" N, 13° 16' 40" E, 3 m a.s.l.), within the polluted site called "Lagoon of Grado and Marano and adjacent rivers", which has been included in the national clean-up priority list of polluted sites (Italian Ministerial Decree 468/2001). The study area is heavily contaminated by pyrite wastes, discharged in the past (1938-1970s) next to the Caffaro chemical plant as by-products of sulphur extraction.

Pyrite ore mainly contains pyrite (FeS₂), lesser amounts of chalcopyrite (CuFeS₂), sphalerite (ZnS), and magnetite (Fe₃O₄), and roasting (800 °C) had made various trace elements available in the remaining waste. The cinders extended for about a depth of 0.7 m over a deep horizon of clay, and had been covered with unpolluted gravelly soil 0.15-0.2 m thick. The cinders were largely devoid of organic matter because of the roasting process, and had high bulk density (1.65) and poor nutritional status, with pH 7.3 and relatively low electrical conductivity (0.3 S m⁻¹). The site had been colonised by spontaneous flora, the

residues of which had accumulated over time. A brief characterisation of the site with its resident flora was given by Coletto et al. (2006).

The total concentrations of metals were measured in cinders before and after soil tillage. Samples ($n=3$) were air-dried and sieved (2 mm), and about 1 g DW was microwave acid-digested following EPA method 3051 (USEPA, 1995a). Before analysis by ICP-OES (SPECTRO CirOS Vision EOP, SPECTRO Analytical Instruments GmbH & Co. KG, Kleve, D), the samples were filtered (0.45 μm PTFE) and diluted to 25 mL with distilled water.

DTPA-extractable fractions of Cu, Cd, Pb and Zn in the cinders were also analysed. Analysis was performed in triplicate on 50 g of homogenised dried cinders by extraction through a 100-mL solution of DTPA (1.97 g L⁻¹), calcium chloride bihydrate (1.46 g L⁻¹), and triethanolamine (14.92 g L⁻¹), pH 7.3, and shaken for 2 hours (60 cycles/min). ICP-OES analysis followed centrifugation (5 min, 5000 rpm).

Trial set up

The trial was conducted during summer 2005 directly in the polluted site. The experimental location has a temperate climate (annual temperature 13.5 °C, precipitation 1200 mm). In 2005, these mean annual parameters were lower than the mean (12.7 °C, 1100 mm), and the growing season (April-September) occurred at a mean temperature of 19 °C and 730 mm of precipitation, 330 mm of which fell in the period April-July. Before cultivation, a preliminary coarse sieving of the top carry-over soil was made, in order to remove the largest size fraction of the skeleton (> 50 mm diam.). Despite this, the tilled layer still had large amounts of skeleton (~60%).

Four herbaceous crop species were sown in two different types of soil tillage (i.e., ploughing and subsoiling, 0.35 m depth) with 3 replicates in a split-plot experimental design. Essentially, ploughing involves a more thorough mixing of soil than subsoiling, which simply rips the surface. Ploughing was intended to dilute the cinders with the unpolluted top soil, and subsoiling to allow roots to encounter a better initial habitat. Because of excavation (for sieving) and shift of cinders during tillage, marked

contamination of cinders was also verified in the top layer of the subsoiled treatment (see below).

Sunflower (*Helianthus annuus* L., cv. Barolo), Italian ryegrass (*Lolium multiflorum* Lam., cv. Lenore), alfalfa (*Medicago sativa* L., cv. Europe) and fodder radish (*Raphanus sativus* L. var. *oleiformis* Pers., cv. Siletta nova) were sown on April 4 in plots of 2×5 m. Sunflower was sown in rows and the other species broadcast. Because of the inhospitality of the substrate, we adopted a seed density about double that of the standard reference for agricultural use, the amount of viable seeds per m² being 17 (sunflower), 200 (fodder radish), 2,200 (Italian raygrass) and 4,200 (alfalfa).

The four species were also grown in a ploughed loamy soil on the experimental farm at Legnaro (Padova, 45°21' N, 11°58' E, 12 m a.s.l.) (uncontaminated reference), specifically in order to compare species shoot and root growth. Before sowing, the soil was fertilised with 100 kg of N, P₂O₅ and K₂O per hectare, and then harrowed. Plots were irrigated when rainfall was insufficient for crop demand during summer. In the May-June period, 30 mm of water were given twice, through a sprinkling system.

Volumetric root length density (RLD) and shoot dry weight were recorded at the flowering stage, in comparison with the reference uncontaminated soil.

Shoot measurements

Leaf chlorophyll content of adult plants was indirectly measured in mid-June with a SPAD 502 chlorophyll meter (Konica-Minolta, Hong Kong) in the youngest fully developed leaves of ten plants in each plot (4 measurements per leaf on average). Measurements were made at the polluted site in comparison with the uncontaminated reference, as an indicator of plant nutritional status and possible damage due to trace metals.

At the flowering stage, on June 22 (radish) and July 6 (other species), shoot samples were harvested from 0.4 m² of each plot and weighed after oven-drying (105 °C, 24 hours) in order to estimate above-ground productivity. At Legnaro (uncontaminated reference) the samples were collected about 10 days earlier than in the polluted site, due to anticipated flowering.

Metal concentrations in shoots was determined on about 0.4 g of dried biomass. Samples were added with 5 mL of nitric acid (70% v/v) and 1.5 mL hydrogen peroxide (30% v/v) and digested in a microwave oven, following EPA method 3052 (USEPA, 1995b). They were then filtered (0.45 µm PTFE) and diluted to 25 mL with distilled water. Arsenic and the above-limit (Cd, Co, Cu, Pb, and Zn) and some below-limit toxic metals (Cr, Mn, Ni) were detected by ICP-OES analysis.

In order to assess overall capacity to concentrate metals, a summation of moles of the nine above-mentioned elements was calculated. Contaminant offtake was calculated by multiplying the above-ground productivity (dry weight) by the concentration of each metal.

Root analysis

At harvest, within the same 0.4-m² sampling area for shoot analysis, two soil cores (70 mm diam.) per plot were collected by the auger sampling method at a depth of 0.5 m (Torviscosa) and 1 m (reference at Legnaro). Each core was subdivided into 100-mm long sub-samples in order to represent the root profile. Separation of roots from soil particles was facilitated by the addition of oxalic acid solution (2% w/v) in a beaker. Roots were separated by flotation by means of a hydraulic centrifugation device, collected in a 500-µm mesh sieve, as described by Oliveira et al. (2000), and stored at 4 °C in an ethanol solution (10% v/v) until analysis. One-bit 375-DPI (i.e., 14.8 pixel mm⁻¹) TIFF-format images of roots were acquired by digital scanning on a flatbed scanner (HP ScanJet 4C, HP, Palo Alto, CA, USA). Measurements of root length were performed by KS 300 image analysis software (Carl Zeiss Vision GmbH, München, Germany), following the procedure of Vamerali et al. (2003). Automatic discrimination of roots against extraneous matter was based on the object shape, suitable root recognition being obtained by adopting an Elongation Index value (i.e., perimeter²/area) of >75 and an object area of >8 pixel.

Root length was obtained by the function *FbL* (fibrelength), based on the values of the perimeter (*P*) and area (*A*) of the digital objects, as follows:

$$FbL = \frac{P\sqrt{P-4A}}{4}$$

Output from image analysis, given as length of roots on the scanner bed, was converted to volumetric density (RLD, root length density, cm cm^{-3}) by taking the size of core sub-samples into account.

The dry weight of root samples was determined after drying (105°C , 24 hours) and metal content was analysed by ICP-OES as described for shoot tissues. Specific root length (SRL) was calculated as the ratio between the length and dry weight of root samples.

Statistical analysis

Statistical analysis of growth parameters, accumulation of metals and correlations was carried out by Statgraphics software (Manugistic Inc., Rockville, MD – USA). After checking normality of data, ANOVA and LDS tests ($P \leq 0.05$) were used to evaluate differences among means for both species and tillages. In analysis, the uncontaminated reference was considered for shoot and root growth only.

Principal component analysis, discriminant analysis and cluster analysis were applied to growth and metal concentration data to describe the characteristics of dissimilarity among species and treatments, by statistical analysis software XLStat (Addinsoft SARL, Paris, France). In dendrograms, the maximum level of homogeneity within groups was calculated with the method of Calinski and Harabasz (1974) and robustness of cluster classification was tested through the stability of the cophenetic coefficient correlation (Sokal and Rohlf, 1962).

Results

Soil analysis

Analysis of cinders showed dangerous contamination by arsenic and five trace metals, i.e., Cd, Co, Cu, Pb and Zn (Table 1), the concentrations of which were considerably in excess of Italian guideline values (IGV) for agricultural uses (Italian Ministerial Decree 156/2006). Metals exceeded the IGV 45 times for As, 23 for Cu, 16 for Zn, 5 for Co and Pb, and about 3 for Cd. Contamination by As and Cu was particularly high, exceeding the less restrictive IGV for industrial uses of soil.

Soil tillage markedly reduced the metal concentrations in pyrite wastes, the extent depending on type of management. Metal contents were roughly halved with ploughing, and reduced to one-third with subsoiling. For all the above-limit metals, subsoiling led to concentrations significantly ($P \leq 0.05$) lower than those for ploughing, about 30% (total) and 35% (DTPA-extractable). In subsoiling, contamination by Cd and Pb was even lower than the IGV.

Irrespective of total content, the bioavailable DTPA-extractable fraction of metals was very similar between tillage systems, being 4% for Zn, 11% for Cd and Pb, and 17% for Cu. In the more severe contamination conditions (ploughing), Ni (10 mg kg^{-1}), Cr (15 mg kg^{-1}) and Mn (187 mg kg^{-1}) were not elevated.

After tillage, fine soil ($< 2 \text{ mm diam.}$) contained a high fraction of sand ($>65\%$), a still subalkaline pH (~ 7.5) and appreciable contents of organic matter ($\text{TOC} > 14 \text{ g kg}^{-1}$) (Table 2). The value of Fe in cinders ($\sim 80\%$) was reduced by tillage to approximately the same extent as trace metals, with higher contents in ploughing than in subsoiling (31% vs. 19%). The wastes had various chemical parameters comparable with the reference uncontaminated loamy soil, but higher electrical conductivity, S content and exchangeable K, and lower available P.

Table 1. Total and DTPA-extractable contents $\pm$ S.E. (n=3) of above-limit trace elements in cinders before and after tillage in comparison with reference uncontaminated loamy soil and maximum permitted concentrations (Italian Ministerial Decree 156/2006, green and residential uses).

n.d. = not determined.

Metal	Legal limit (mg kg ⁻¹)	Pyrite cinders (mg kg ⁻¹)	Pyrite cinders Ploughing		Pyrite cinders Subsoiling		Reference soil	
			Total (mg kg ⁻¹)	DTPA-extractable (mg kg ⁻¹)	Total (mg kg ⁻¹)	DTPA-extractable (mg kg ⁻¹)	Total (mg kg ⁻¹)	DTPA-extractable (mg kg ⁻¹)
As	20	892 $\pm$ 15	423 $\pm$ 46	n.d.	292 $\pm$ 37	n.d.	15.5 $\pm$ 0.86	-
Cd	2	5.1 $\pm$ 0.2	2.67 $\pm$ 0.42	0.31 $\pm$ 0.08	1.67 $\pm$ 0.20	0.16 $\pm$ 0.02	0.38 $\pm$ 0.03	0.09 $\pm$ 0.01
Co	20	102 $\pm$ 8	42 $\pm$ 5.00	n.d.	29 $\pm$ 3.54	n.d.	7.95 $\pm$ 0.34	-
Cu	120	2,726 $\pm$ 199	1,719 $\pm$ 228	291 $\pm$ 39	1,103 $\pm$ 155	190 $\pm$ 25	31.1 $\pm$ 2.09	4.51 $\pm$ 0.28
Pb	100	495 $\pm$ 30	118 $\pm$ 8.53	16 $\pm$ 1.93	90 $\pm$ 15	10 $\pm$ 2.01	17.9 $\pm$ 1.33	2.96 $\pm$ 0.25
Zn	150	2,410 $\pm$ 241	860 $\pm$ 66	30 $\pm$ 5.32	674 $\pm$ 46	24 $\pm$ 1.97	79.5 $\pm$ 4.10	1.21 $\pm$ 0.13

Table 2. Main physical and chemical parameters ($\pm$ S.E.) of cinders and reference uncontaminated loamy soil (n=3).

Parameter		Method	Uncontaminated reference	Pyrite cinders Ploughing	Pyrite cinders Subsoiling
Sand (2–0.05 mm)	(%)		43.1 $\pm$ 2.91	65.2 $\pm$ 0.67	68.4 $\pm$ 1.23
Silt (0.05–0.002 mm)	(%)		48.2 $\pm$ 2.56	29.3 $\pm$ 0.00	25.5 $\pm$ 1.18
Clay (<0.002 mm)	(%)		8.67 $\pm$ 1.36	5.50 $\pm$ 0.67	6.10 $\pm$ 0.67
Total organic carbon	(g kg ⁻¹)	Walkey & Black	12.6 $\pm$ 2.02	14.6 $\pm$ 0.71	16.6 $\pm$ 0.62
Soil organic matter	(g 100g ⁻¹)	Walkey & Black	2.17 $\pm$ 0.35	2.52 $\pm$ 0.12	2.85 $\pm$ 0.11
pH		H ₂ O	7.80 $\pm$ 0.02	7.56 $\pm$ 0.02	7.49 $\pm$ 0.06
Cation exchange capacity	(Cmol ⁺ kg ⁻¹)	BaCl ₂	14.5 $\pm$ 1.06	17.1 $\pm$ 0.66	15.9 $\pm$ 0.90
Electrical conductivity	(S cm ⁻²)	ISO 11265	0.03 $\pm$ 0.001	0.31 $\pm$ 0.01	0.29 $\pm$ 0.01
N (Total)	(g kg ⁻¹)	Kjeldahl	0.6 $\pm$ 0.12	0.5 $\pm$ 0.03	0.7 $\pm$ 0.09
P (Total)	(mg kg ⁻¹)	USEPA 3051	594 $\pm$ 53.1	203 $\pm$ 3.32	158 $\pm$ 9.18
P (Available)	(mg kg ⁻¹)	Olsen	33.1 $\pm$ 10.6	12.7 $\pm$ 1.93	7.90 $\pm$ 0.99
K (Total)	(g 100g ⁻¹)	USEPA 3051	1.60 $\pm$ 0.04	0.33 $\pm$ 0.01	0.34 $\pm$ 0.04
K (Exchangeable)	(mg kg ⁻¹)	BaCl ₂ TEA (ISO 11260)	62.8 $\pm$ 5.33	153 $\pm$ 6.74	131 $\pm$ 16.4
S (Total)	(g kg ⁻¹)	USEPA 3051	0.19 $\pm$ 0.001	3.38 $\pm$ 0.29	3.22 $\pm$ 0.25
Fe (Total)	(g kg ⁻¹)	USEPA 3051	20.3 $\pm$ 0.81	310 $\pm$ 40	195 $\pm$ 33
Fe (Available)	(mg kg ⁻¹)	DTPA BaCl ₂ TEA (ISO/DIS 14870)	18.3 $\pm$ 1.10	24.0 $\pm$ 1.24	26.3 $\pm$ 2.40

Shoot and root growth

At the scheduled harvest time, all species had reached the flowering stage, except radish, which had already entered the pod filling stage. In wastes, seed germination and plant growth were appreciable, allowing a satisfactory canopy cover. Compared with the uncontaminated reference, retarded flowering was symptomatic of reduced growth potential. From visual evaluation, good above-ground growth was expected by radish and sunflower, and less by alfalfa and ryegrass, and the rates were confirmed by above-ground productivity (Fig. 1). Biomass reduction was maximum in *L. multiflorum* (-91%) and minimum in *R. sativus* (-71%).

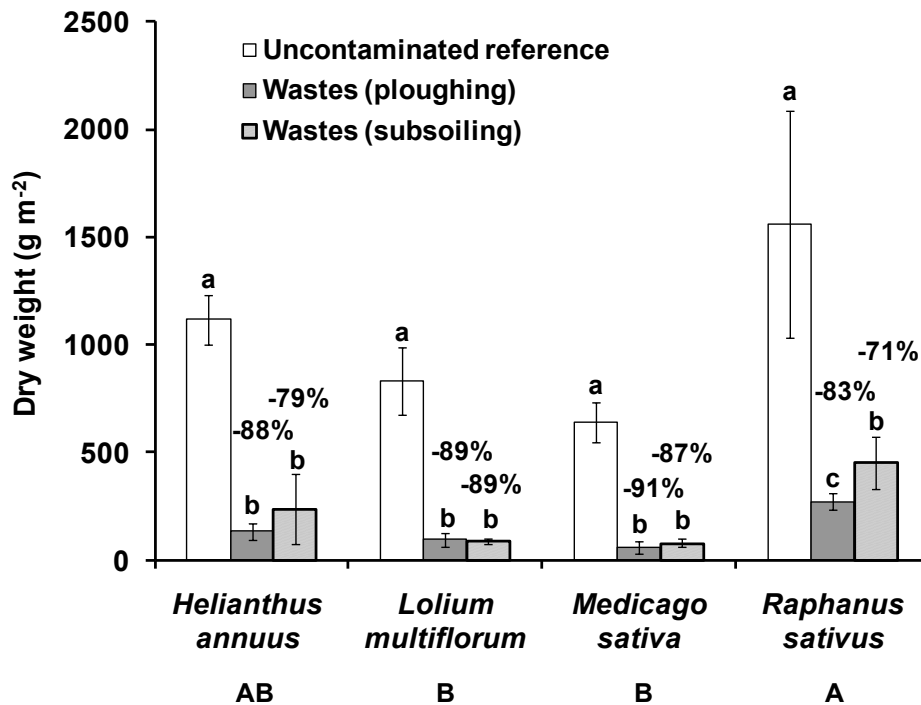


Figure 1. Shoot dry weight ($\pm$ S.E., $n = 3$) at flowering stage of species in pyrite wastes in comparison with uncontaminated reference. Percentages: reduction for ploughing and subsoiling from controls. Small letters: statistically significant differences between treatments within same species (LSD test, $P \leq 0.05$). Capital letters: statistically significant differences between species for wastes only.

Growth differences recorded between species in the uncontaminated soils were reflected in their growth in the wastes, in the order radish $\geq$ sunflower $\geq$ ryegrass = alfalfa (main effect). *Raphanus sativus* reached higher biomass ($P \leq 0.05$) in the weaker contamination conditions of subsoiling than in ploughing (450 vs. 270 g m⁻²), whereas this was only a trend in *H. annuus* and *M. sativa* (Fig. 1).

The better adaptation of radish and sunflower to pyrite wastes was confirmed by their normal leaf chlorophyll content, SPAD values being comparable with the uncontaminated reference, irrespective of soil management (data not shown). Instead, ryegrass and alfalfa had values 35% and 20% lower than controls, respectively.

In wastes, root growth and deepening was markedly limited in all species. Maximum depth was about 0.3 m – much lower than that of 0.7-1 m in controls (Fig. 2).

The mean volumetric root length density (RLD) was higher with subsoiling than with ploughing (main effect: 5.7 vs. 4.9 cm cm⁻³). ($P \leq 0.05$). Compared with controls, root length was reduced by 50% in radish and alfalfa, 60% in sunflower and 70% in ryegrass. Subsoiling was clearly beneficial for root growth in sunflower and alfalfa, but was less effective in the other species. Ryegrass reached the maximum and alfalfa the minimum RLD, producing 7.3 and 4.2 cm cm⁻³, respectively, as averages of the explored profiles. Much of the root length was confined in the 0.1-m top soil (Fig. 3). There was no significant ‘species $\times$ tillage’ interaction, although radish only had higher RLD in ploughing than subsoiling, and both were greater than controls. Root distribution was similar between tillages in sunflower and alfalfa, unlike radish and ryegrass which benefited from subsoiling by showing a greater fraction of length in deep layers (Fig. 3).

Root length was generally well correlated with root biomass (dry weight), the minimum coefficient of determination (R^2) being 0.52 (sunflower) and the maximum 0.85 (alfalfa) in both soil managements, a fact which implied a stable specific root length (SRL). This was true for tillages, but significant differences ($P \leq 0.05$) were revealed among species, ryegrass showing much higher efficiency than the other species (150 vs. 100 m g⁻¹).

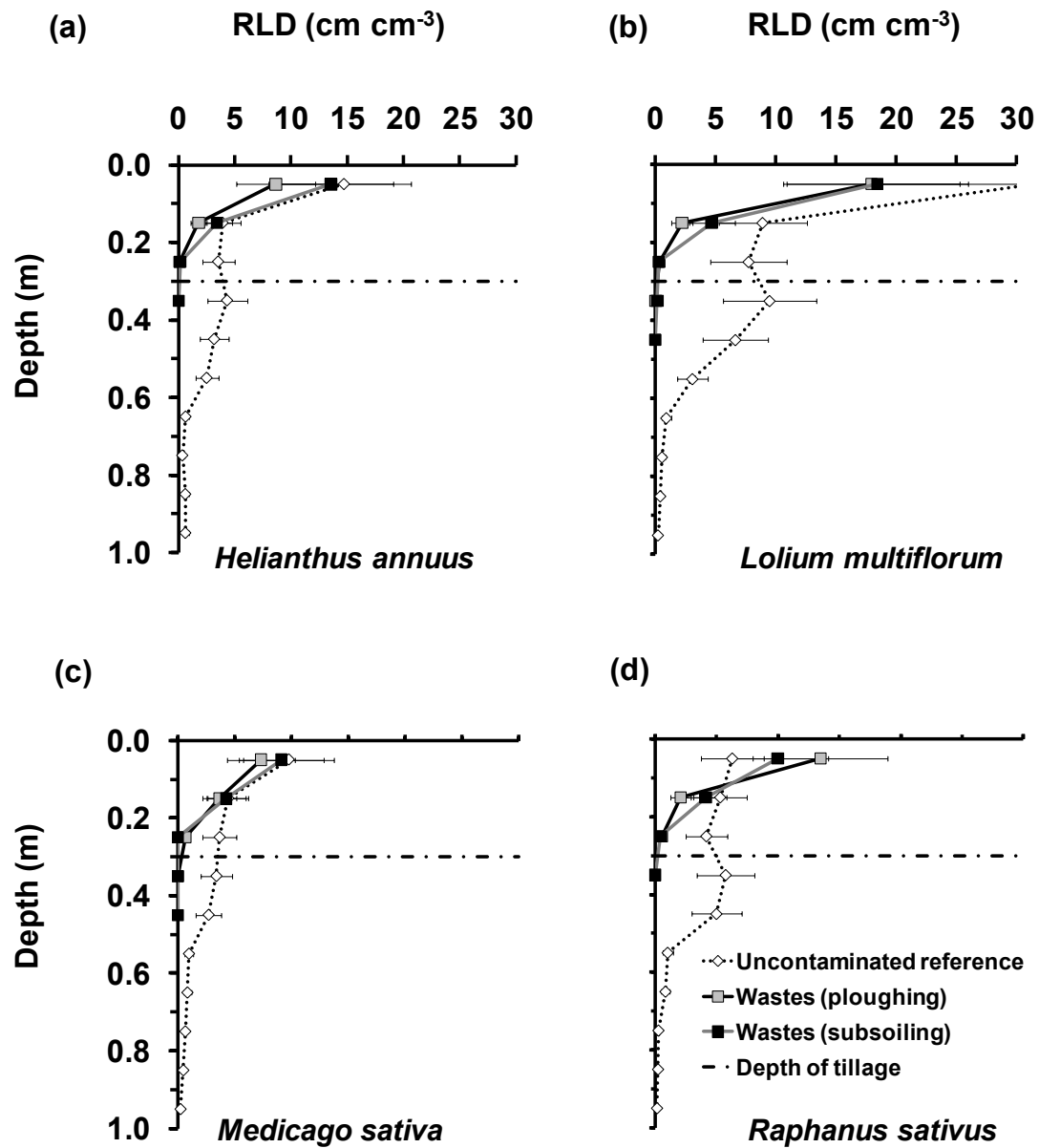


Figure 2. Patterns of root length densities (RLD) of species in pyrite wastes in comparison with uncontaminated reference. Horizontal dot-dashed line: depth of tillage. Horizontal bars: standard errors ($n = 6$).

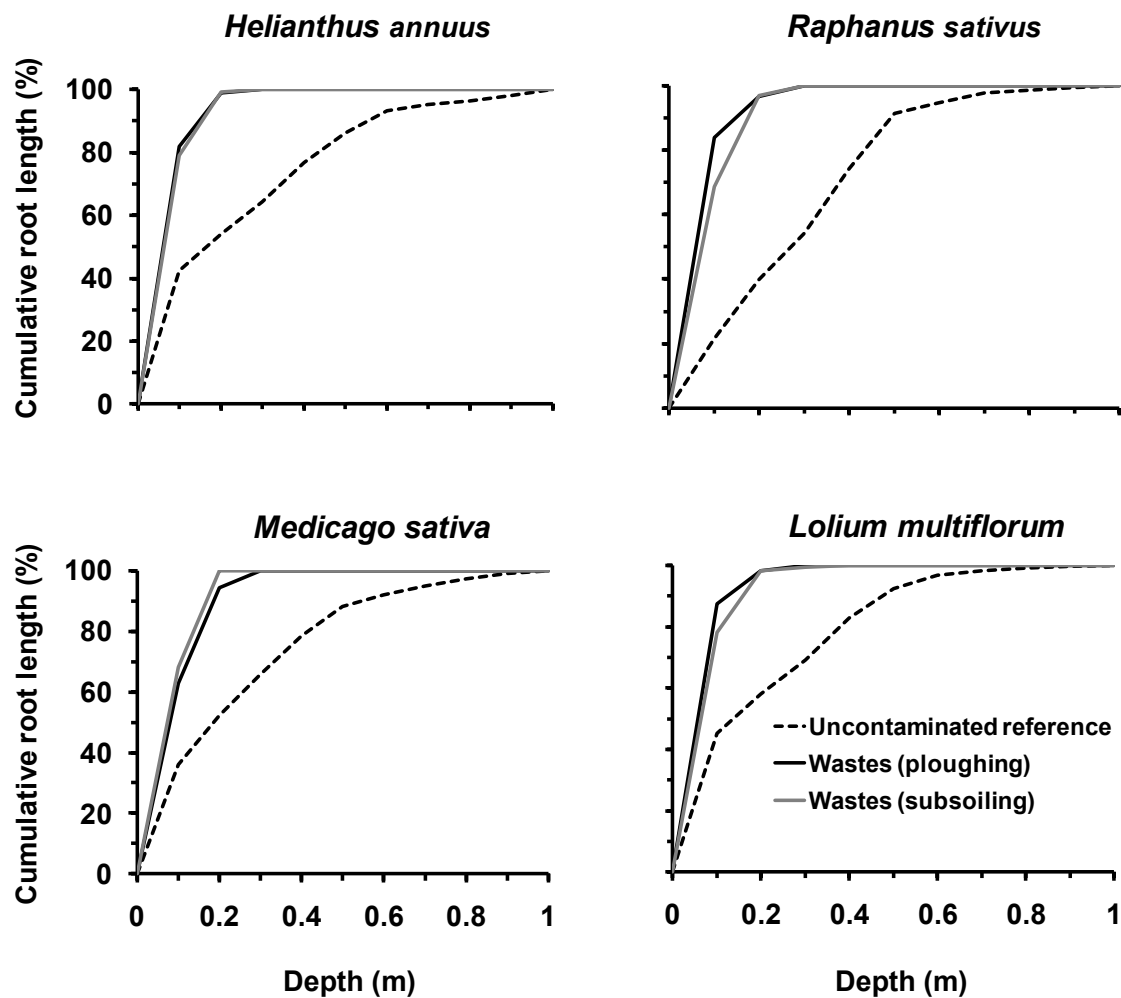


Figure 3. Cumulative root length distribution along profile of species in wastes compared with uncontaminated reference.

Metal accumulation in plant tissues

ICP analysis of shoot tissues revealed a tendency toward greater accumulation for the above-limit metals in ploughing than in subsoiling, but this was only significant for Zn (111 vs. 68 mg kg⁻¹) ($P \leq 0.05$) (Table 3). Low concentrations were reported for Cd and Co, values being 0.4 and 0.33 mg kg⁻¹ (mean of tillages), respectively. Arsenic was rarely found in sunflower and was relatively low (3.4 mg kg⁻¹), like Pb (1.3 mg kg⁻¹), whereas Cu was appreciable (24 mg kg⁻¹). Concentration of metals was negatively correlated with P content, particularly with As ($R^2 = 0.18$) and Zn ($R^2 = 0.42$).

The higher total and DTPA-extractable metals in ploughing were associated with greater accumulation of Cd, Co and Zn in sunflower and Cd in radish, but this did not give rise to substantial differences among species in terms of overall accumulation (summation of 9 metal moles). The opposite occurred in subsoiling, which caused the overall accumulation of ryegrass to be about 3 times greater than in sunflower and radish, with high Cd, Pb and Zn.

Trace element concentrations were much higher in fine roots than in above-ground tissues, with higher values again in ploughing than subsoiling for all metals – except Pb – and their summation. As in shoots, the summation of 9 metal concentrations differed between species in subsoiling only, but in the opposite order (sunflower > ryegrass). This reflected the generally small translocation factor (TF), i.e. the shoot-to-root metal concentration ratio, which varied only among species (Fig. 4). For the 9 elements combined, TF was highest in ryegrass (9 vs. 4 % of the other species), whereas among elements the best TF were found for Cd (27%) and Zn (13%) and worst for Cu (1% only), Pb and As (~2%).

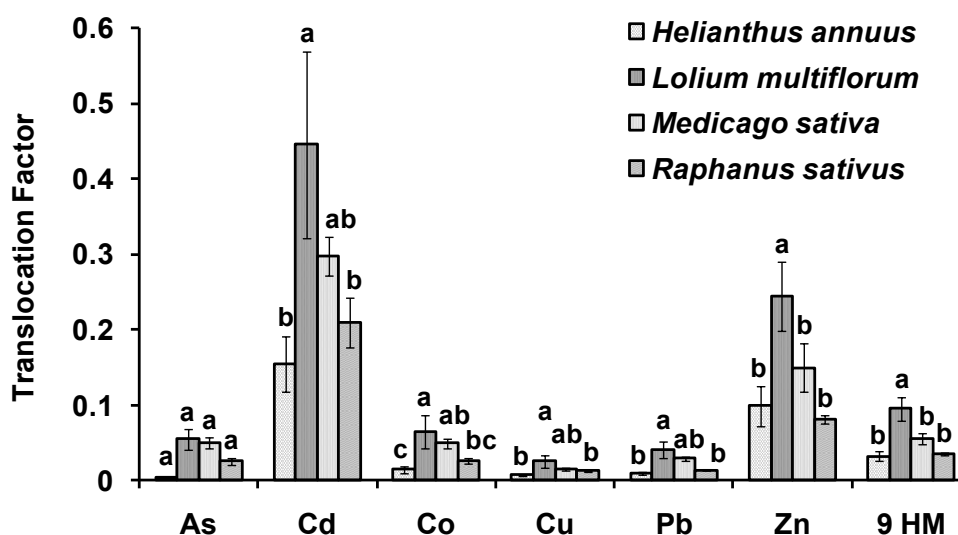


Figure 4. Shoot-to-root metal concentration ratio (translocation factor) of species grown in pyrite wastes ($\pm$ S.E., $n = 6$) for various metals and summation of 9 trace elements (9 HM). Letters: statistically significant differences among species within same metal (LSD test, $P \leq 0.05$).

In terms of mass balance, the overall removal of metals (above-ground biomass, 9 trace elements) did not differ between soil managements, and was significantly higher in *R. sativus* than in *H. annuus* and *L. multiflorum* (Fig. 5). Removals followed the order fodder radish > Italian ryegrass > sunflower > alfalfa, i.e., 33, 21, 17 and 9 mg m⁻² respectively, with Zn (62%) and Cu (18%) as main contaminants harvested (Fig. 5).

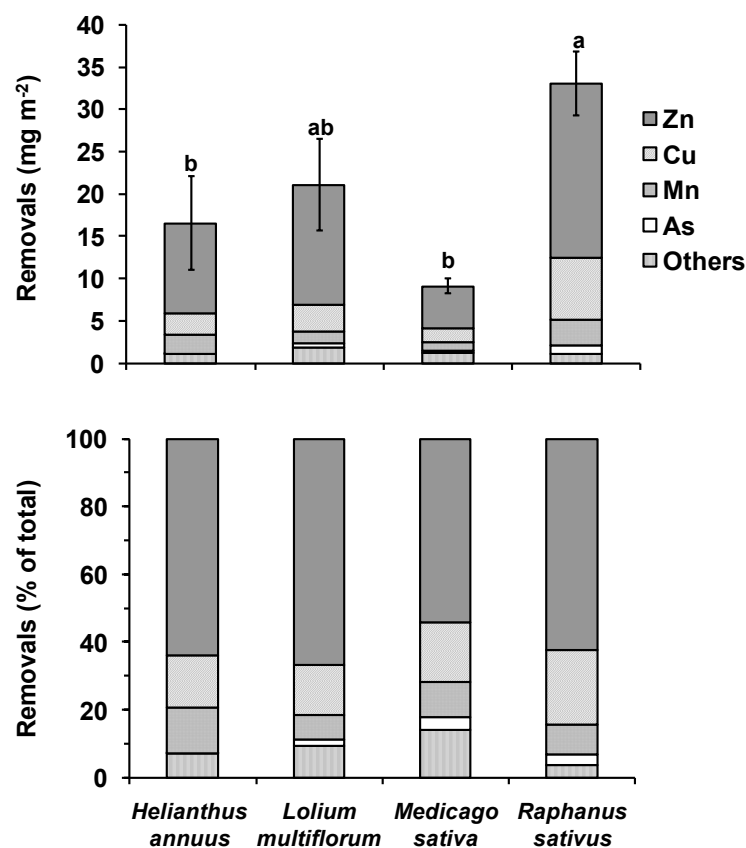


Figure 5. Metal removals ($\pm$ S.E) and their fractions of species grown in pyrite wastes (n=3). Letters: statistically significant differences among species (LSD test, $P \leq 0.05$).

Table 3. Concentrations $\pm$ S.E. (n=3) in shoot and fine root tissues of species under two tillage systems (LSD test, $P \leq 0.05$). Small letters: differences among species within same tillage. Capital letters: for each metal, statistically significant differences for “Treatment” main effect.

Treatment	Species	As (mg kg ⁻¹)	Cd (mg kg ⁻¹)	Co (mg kg ⁻¹)	Cu (mg kg ⁻¹)	Pb (mg kg ⁻¹)	Zn (mg kg ⁻¹)	9 HM (mmol kg ⁻¹)
<i>Shoot</i>								
Ploughing	<i>H. annuus</i>	0.15 $\pm$ 0.2 c	0.42 $\pm$ 0.08 a	0.19 $\pm$ 0.04 c	14 $\pm$ 3.6 b	0.48 $\pm$ 0.13 c	89 $\pm$ 15 a	1.9 $\pm$ 0.27 a
	<i>L. multiflorum</i>	3.9 $\pm$ 0.7 b	0.42 $\pm$ 0.05 a	0.41 $\pm$ 0.08 ab	30 $\pm$ 5.8 a	1.7 $\pm$ 0.18 a	160 $\pm$ 42 a	3.4 $\pm$ 0.7 a
	<i>M. sativa</i>	6.9 $\pm$ 0.4 a	0.45 $\pm$ 0.03 a	0.52 $\pm$ 0.05 a	30 $\pm$ 3.3 a	2.2 $\pm$ 0.19 a	119 $\pm$ 36 a	2.7 $\pm$ 0.61 a
	<i>R. sativus</i>	3.3 $\pm$ 0.4 b	0.55 $\pm$ 0.07 a	0.29 $\pm$ 0.04 bc	25 $\pm$ 0.55 ab	1.1 $\pm$ 0.13 b	75 $\pm$ 10 a	1.8 $\pm$ 0.19 a
	MEAN	3.6 $\pm$ 0.8 A	0.46 $\pm$ 0.03 A	0.35 $\pm$ 0.04 A	25 $\pm$ 2.5 A	1.4 $\pm$ 0.21 A	111 $\pm$ 16 A	2.5 $\pm$ 0.29 A
Subsoiling	<i>H. annuus</i>	n.d.	0.15 $\pm$ 0.01 b	0.05 $\pm$ 0.02 a	11 $\pm$ 1.3 a	0.13 $\pm$ 0.13 b	34 $\pm$ 2 b	0.94 $\pm$ 0.08 b
	<i>L. multiflorum</i>	5.4 $\pm$ 3.5 a	0.58 $\pm$ 0.22 a	0.58 $\pm$ 0.34 a	37 $\pm$ 19 a	2.3 $\pm$ 1.2 a	126 $\pm$ 23 a	3 $\pm$ 0.77 a
	<i>M. sativa</i>	4.2 $\pm$ 1.6 a	0.33 $\pm$ 0.07 ab	0.37 $\pm$ 0.12 a	23 $\pm$ 5.9 a	1.42 $\pm$ 0.35 ab	64 $\pm$ 14 b	1.7 $\pm$ 0.35 ab
	<i>R. sativus</i>	3.0 $\pm$ 1.3 a	0.27 $\pm$ 0.04 ab	0.21 $\pm$ 0.04 a	18 $\pm$ 2.7 a	0.58 $\pm$ 0.29 ab	48 $\pm$ 7.5 b	1.2 $\pm$ 0.17 b
	MEAN	3.2 $\pm$ 1.1 A	0.33 $\pm$ 0.07 A	0.30 $\pm$ 0.10 A	22 $\pm$ 5.2 A	1.1 $\pm$ 0.38 A	68 $\pm$ 12 B	1.7 $\pm$ 0.30 B
<i>Roots</i>								
Ploughing	<i>H. annuus</i>	142 $\pm$ 13 a	2.8 $\pm$ 0.88 a	9.9 $\pm$ 1.3 a	2,266 $\pm$ 261 a	75 $\pm$ 13 a	669 $\pm$ 62 b	49 $\pm$ 5.4 a
	<i>L. multiflorum</i>	81 $\pm$ 8.7 b	1.3 $\pm$ 0.13 b	8 $\pm$ 1.2 a	1,691 $\pm$ 266 a	51 $\pm$ 5.4 a	659 $\pm$ 97 b	40 $\pm$ 5.7 a
	<i>M. sativa</i>	127 $\pm$ 22 ab	1.5 $\pm$ 0.25 ab	9.6 $\pm$ 1.6 a	1,852 $\pm$ 285 a	71 $\pm$ 12 a	629 $\pm$ 93 b	42 $\pm$ 6.2 a
	<i>R. sativus</i>	141 $\pm$ 20 a	1.6 $\pm$ 0.42 ab	11 $\pm$ 1.4 a	2,280 $\pm$ 207 a	79 $\pm$ 12 a	931 $\pm$ 96 a	54 $\pm$ 4.8 a
	MEAN	123 $\pm$ 9.3 A	2.0 $\pm$ 0.27 A	9.6 $\pm$ 0.68 A	2,022 $\pm$ 131 A	69 $\pm$ 5.5 A	722 $\pm$ 48 A	46 $\pm$ 2.9 A
Subsoiling	<i>H. annuus</i>	114 $\pm$ 22 a	1.4 $\pm$ 0.18 a	10 $\pm$ 2 a	2,227 $\pm$ 477 a	61 $\pm$ 12 a	665 $\pm$ 98 a	49 $\pm$ 9.6 a
	<i>L. multiflorum</i>	76 $\pm$ 11 a	1.2 $\pm$ 0.23 a	7.3 $\pm$ 1.5 a	1,157 $\pm$ 192 b	50 $\pm$ 7.8 a	555 $\pm$ 75 a	30 $\pm$ 4.9 b
	<i>M. sativa</i>	91 $\pm$ 7.2 a	1.1 $\pm$ 0.1 a	8.3 $\pm$ 0.91 a	1,645 $\pm$ 270 ab	54 $\pm$ 6.2 a	587 $\pm$ 46 a	38 $\pm$ 4.9 ab
	<i>R. sativus</i>	110 $\pm$ 12 a	1.8 $\pm$ 0.37 a	8.9 $\pm$ 0.98 a	1,430 $\pm$ 176 ab	67 $\pm$ 5.7 a	602 $\pm$ 42 a	35 $\pm$ 3.4 ab
	MEAN	98 $\pm$ 7.3 B	1.4 $\pm$ 0.13 B	8.8 $\pm$ 0.70 B	1,615 $\pm$ 163 B	58 $\pm$ 4.1 A	602 $\pm$ 33 B	38 $\pm$ 3.2 B

Species classification and principal component analysis

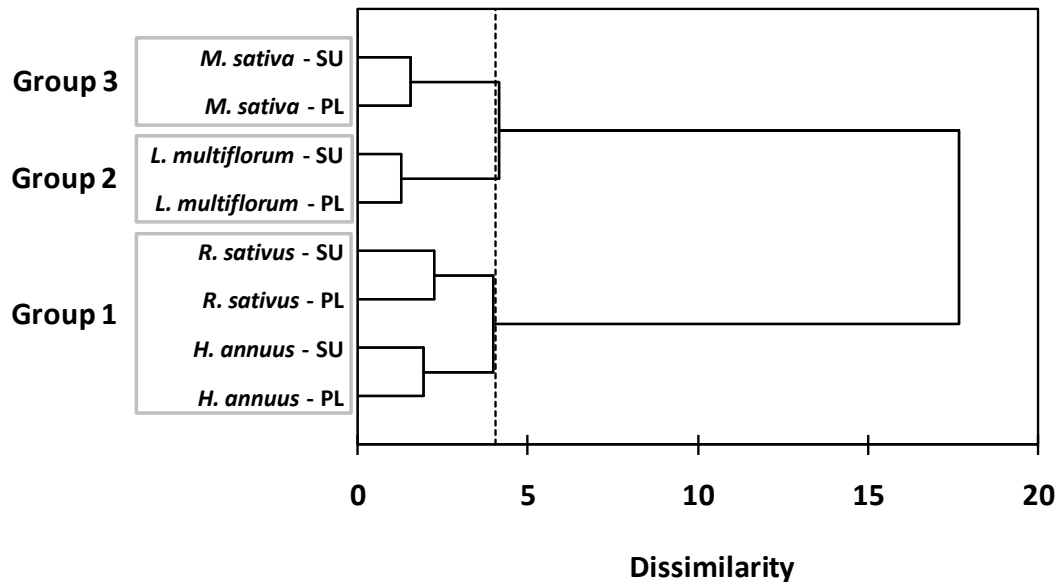
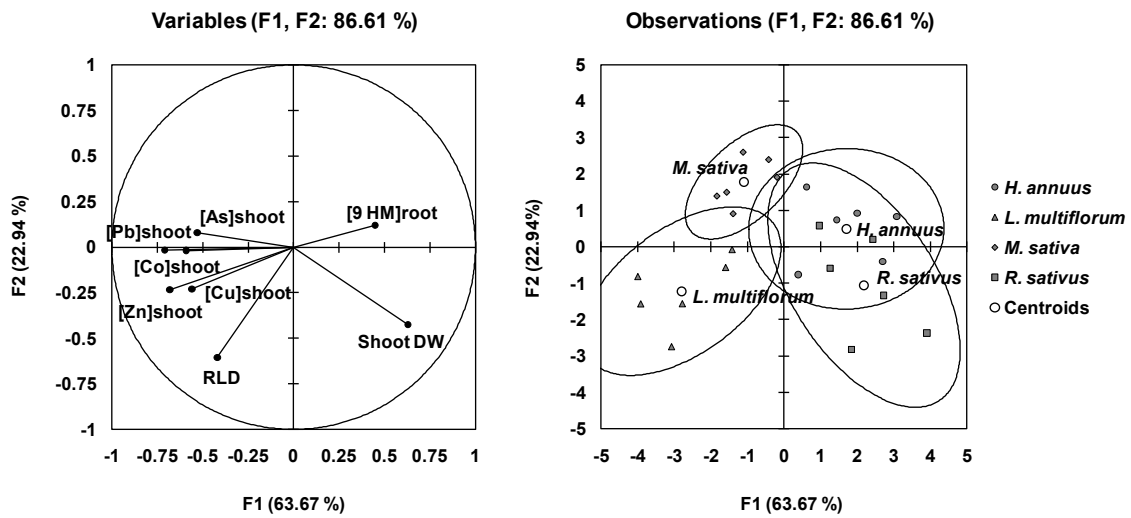


Figure 6. Cluster classification of species under ploughing (PL) and subsoiling (SU). Variables analysed: shoot biomass, RLD, As, Co, Cu, Pb and Zn concentrations in shoot, and summation of concentrations of 9 trace metals in roots. Vertical dashed line: maximum level of homogeneity within groups.

When shoot biomass, RLD, As, Co, Cu, Pb and Zn concentrations in shoot, and summation of 9 trace metals in roots were considered in cluster analysis, the clearcut subdivision of species into three groups was revealed (Fig. 6). Irrespective of soil management, group 1 comprised fodder radish and sunflower, having higher above-ground yield and generally lower concentrations of metals. Group 2 contained ryegrass, which had the highest metal concentrations and root densities, and low biomass. Alfalfa belonged to group 3 and had intermediate values of biomass and metal contents. The cophenetic correlation coefficient calculated for dendrograms using average, complete and proportional linkage clustering was stable and very high (>0.84), statistically supporting the above species classification. These results were confirmed by principal component analysis (PCA) and discriminant analysis (Fig. 7). PCA identified two significant dummy variables which explained 87% of variability. The first factor (F1) explained 64% of the variance and

was correlated (loadings>0.5) with shoot biomass and its metal concentrations; the second factor (F2) accounted for 23% of variance and was correlated with root length density. On the basis of the opposite direction of vectors (Fig. 7, left), negative correlations were more evident for pairs of variables ‘shoot As–shoot biomass’, ‘shoot Zn and Cu–overall metals in roots’ and ‘RLD–overall metals in roots’. Correlations between root density and Cu and Zn in shoots were positive.

Overlaps among ellipses and positions of centroids (Fig. 7, right) indicated that species in group 1 (sunflower and radish) had high above-ground productivity, ryegrass high RLD and metal concentration in the harvestable canopy, and alfalfa a good concentration of As in shoots.



Variable	F1	F2
Shoot DW	0.629	-0.43
RLD	-0.42	-0.61
[As]shoot	-0.53	0.08
[Co]shoot	-0.59	-0.012
[Cu]shoot	-0.56	-0.23
[Pb]shoot	-0.71	-0.02
[Zn]shoot	-0.68	-0.234
[9 HM]roots	0.45	0.119

Figure 7. Principal component analysis (top left), discriminant analysis (top right) and loadings of parameters within two main components (Factors 1, 2) for species in wastes. Variables included in analysis: shoot dry weight, root length density (RLD), As, Co, Cu, Pb and Zn concentrations in shoot, and summation of 9 trace elements in roots.

Discussion

Pyrite wastes proved to be an inhospitable substrate for plant growth, as previously reported by Fellet et al. (2007) and Marchiol et al. (2007) in the same site at Torviscosa. The anomalous physical properties of this substrate, together with high Fe, S and multiple trace element contamination, strongly limit plant growth, regardless of species. High bulk density and low permeability to water of the cinders also cause undesirable alternate waterlogging and drought, which may cause marked growth reduction. Although the presence of a top unpolluted soil may be beneficial for plant growth, the question arises regarding how it is managed for cultivation. Maintenance of stratigraphy (subsoiling) leads to lower total and bioavailable metal concentrations in shallow layers than mixing wastes with soil (ploughing), a condition less favourable for metal uptake but more useful for plant growth, as clearly seen in radish. The choice of tillage system was not critical in terms of mass balance of phytoextraction, but subsoiling is preferable, in that it guarantees better canopy cover of the substrate. This can reduce the potential exposure risk to human health, groundwater and other receptors through windblow of fine pyrite particles or leachate movement. Greater plant productivity can also facilitate mechanical management of biomasses, including cutting and harvesting operations after drying in the open air.

Species selection is essential for enhancing phytoremediation. *Raphanus sativus* and *H. annuus* were the highest biomass-yielding species, but only fodder radish reached the highest metal offtakes – a partially expected result, as the *Brassicaceae* family is widely recognised as including efficient species for phytoremediation, many of which are hyperaccumulators (Nanda-Kumar et al., 1995; Ebbs and Kochian, 1997; Ebbs et al., 1997). The biomass reached by radish in wastes was obviously poor, when compared with the uncontaminated reference. Its productivity was similar to that of the small-biomass yielding *Thlaspi caerulescens* (Anderson et al., 1999), although this occurrence should not be considered negatively, as hyperaccumulators cannot grow in pluri-contaminated sites (Quartacci et al., 2003).

Removals were generally poor, reaching 330 g ha⁻¹ as maximum with radish, and involved mainly zinc and copper. Improvements of removals undoubtedly require increases in yields and metal concentrations, and enhancement of translocation factors.

Substantial productivity gains have been obtained with abundant chemical or organic fertilisation of cinders (Marchiol et al., 2007), and the small plant size we observed indicates that plant density can be greatly increased, at least for wide-spaced crops like sunflower. Adequate water drainage and irrigation are further suitable means for stabilising soil moisture in soil and maximising plant growth. The toxic impact of cinders on plant growth was particularly severe in ryegrass and alfalfa, as also shown by their poor growth later in the season, which was insufficient to provide further herbage cuts. Metals can affect pigment contents in green tissues in various ways, as shown in the literature for Cu (Tanyolac et al., 2007; Pourakbar et al., 2007), Pb (Ahmad et al., 2008) and Cd (Shekhawat et al., 2008). The absence of root nodulation and particularly high As in tissues were probably additional constraints in alfalfa.

Metal concentrations in the shoot tissues of our crops were far from reaching hyperaccumulation thresholds (Terry and Bañuelos, 2000), but there is little scope for improvements by means of traditional agricultural practices, as concentrations are commonly negatively correlated with shoot biomass. Uptake was improved in conditions of high levels of metals in soil (ploughing), and this may reduce differences between crops, making species selection less essential. Concentrations in shoots were comparable with those found in sunflower and sorghum by Marchiol et al. (2007) in the same site, confirming stable responses under severe contamination.

At morphological level, better metal translocations were supported by finer roots ($R^2 = 0.21$; $P \leq 0.05$). Reduction of soil pH (Dominguez et al., 2009) and application of chelators (Grčman et al., 2001) have the potential to improve metal uptake and translocation above ground. Nowadays, this option is aided by the availability of less persistent and highly degradable molecules, like NTA and EDDS, which have been efficiently tested in pyrite wastes (Quartacci et al., 2007), particularly for copper uptake (Tandy et al., 2004). The clay layer found at depth in our polluted site is thought to have prevented significant metal leaching until now, and may also act in the case of amendments with chelators.

Fine root growth was less affected than shoot growth, and provided a substantial accumulation sink for most trace elements, particularly Cu, Pb and As. The same result was found in coarse and particularly fine roots of poplars and willow species previously grown at the same site (Vamerali et al., 2009a). The biomass of fine roots in our crops was quite modest, estimates from soil cores not exceeding 20 g m^{-2} (excluding tap roots). These roots provide soil with organic materials through decomposition, but their role in long-term metal stabilisation should be excluded because of the generally fast fine root turnover (Goins and Russelle, 1996; Vamerali et al., 2009c). Despite this, the development of root systems may hold the key to understanding the possibilities and options for phytomanagement of our pyrite wastes. Root length density was related to the concentrations of most metals in the harvestable biomass, a result supporting the great ability of ryegrass in this case. The greater root density of *R. sativus* in top wastes compared with control soils together with great above-ground productivity and metal removals, indicates its lesser sensitivity to pollution. The better root colonisation of this species in the more polluted conditions of ploughing appears to be similar to the behaviour of some hyperaccumulator plants, which show preferential growth in spot-polluted soil volumes (Schwartz et al., 1999). Indeed, radish was conveniently used in our past experiments in a pluri-contaminated soil in Milan (Mosca et al., 2004), showing better shoot growth and root length than controls and better performance than other *Brassicaceae*, such as *Brassica juncea*, *Brassica napus* and *Lepidium sativum*. Further root development is therefore desirable, and promising increases in root length have recently been achieved by soil application of small amounts (0.1 mg kg^{-1}) of humic substances (Bandiera et al., 2009). In pyrite wastes, maximum rooting was 0.3 m, and further increases are probably the hardest problem to solve. The particularly severe conditions of our site are evident in comparison with the results of Keller et al. (2003), who found roots of Indian mustard and maize down to a depth of $\sim 0.7 \text{ m}$ in a Cd-Cu-Zn-contaminated soil. In this regard, the initial characterisation of our polluted site (Coletto, 2006), with its unaltered, unpolluted top layer, revealed a greater number of species with shallow fasciculate roots and more severe selection of tap-rooted ones.

Conclusions

Removal of the most labile fraction of metals, i.e., Bioavailable Contaminant Stripping (BCS) (Hamon and McLaughlin, 1999) of pyrite wastes by means of field crops seems to be a feasible phytomanagement option, at least with some species and trace elements. This *in situ* study provided realistic information on phytoremediation, showing that, among a small range of crops, only *Raphanus sativus* achieved substantial Zn and Cu removals, whereas management of the most zootoxic metals, such as As and Pb, still remains problematic. The great amount of soil As in association with subalkaline pH poses the problem of its high mobility and interaction with P nutrition in plants (Quaghebeur and Rengel, 2004). This occurrence has also been reported in the literature between Cd and several macro-nutrients (P, K, Mg, Fe) (e.g., Cheng et al., 2009).

The much larger variability in shoot metal concentrations of the crops tested here compared with woody species in the same site (Vamerali et al., 2009a) suggests exploiting the potential of other herbaceous species. This would allow the identification of a pool of plants, to be cultivated in association or in rotation, in order to cover the soil permanently and reduce possible damage by parasites.

Besides phytoextraction and energy recovery from biomass combustion, the simple vegetation cover also represents a definite enhancement of the landscape value. For radish in particular, easy dissemination due to this crop's fast growing cycle and pod shattering, means that it may reproduce itself in time. As uptake generally falls after flowering and grains do not accumulate appreciable metals (Vamerali et al., 2009b), self-dissemination may not be the best strategy for phytoremediation. The above-ground productivity we evaluated in this study refers to the flowering stage and does not include successive increases due to grain filling, and identification of species with different growth periods would supply continuous metal uptakes through rapid succession of various species.

Simply covering wastes with a thin layer of imported soil had formerly proven to support an inadequate spontaneous vegetation cover. When an attempt to stabilise wastes with herbaceous crops is made, maintenance of stratigraphy aids their initial growth more

appropriately, although rooting is restricted into the deeper polluted layers. Reduction of metal mobility through soil amendment may be a further option to be tested for reducing the impact on plant growth (Hartley et al., 2004, 2009)

Phytostabilisation of metals through root systems, an occurrence recently claimed for woody species as more realistic than phytoextraction (Vandecasteele et al., 2005; Dickinson et al., 2009), is probably less practicable with crops. Precise quantification of root production and rates of its decomposition over time are required for better understanding of long-term stabilisation, at least in tap-rooted crops.

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Chapter IV. Humic acids affect root characteristics of fodder radish (*Raphanus sativus* L. var. *oleiformis* Pers.) in metal-polluted wastes³

Abstract

In a series of pot trials, fodder radish (*Raphanus sativus* L. var. *oleiformis* Pers.) was cultivated in pyrite cinders heavily polluted by various heavy metals. The effects of application of humic acids (HA) to the cultures were evaluated at various concentrations (0.1 and 1 g kg⁻¹ of cinders) and methods of application (mixed with cinders before sowing; applied on the top of substrate after sowing; foliar spraying), in comparison with untreated controls.

Humic acids increased the bioavailability of various heavy metals, especially when applied at high concentrations. Low concentrations of HA, either mixed with cinders or applied on foliage, slightly enhanced shoot growth. High levels of HA were generally phytotoxic, probably because of the ensuing higher heavy metal concentrations in the shoots. Foliar spraying was partially able to attenuate the phytotoxicity of high dosages of HA, as revealed by the increased rate of leaf expansion.

A negative correlation was found between shoot biomass and concentration of heavy metals, and higher removals were generally associated with smaller doses of HA and foliar application. In particular, mixing 0.1 mg kg⁻¹ of HA before sowing, which enhanced root growth (+46% in length), allowed greater acidification of the cinders and the highest uptake of HM. Within the range of concentrations tested, humic substances led to a reduction in

³ From: Bandiera M., Mosca G., Vamerali T. (2009) Humic acids affect root characteristics of fodder radish (*Raphanus sativus* L. var *oleiformis* Pers.) in metal-polluted wastes. *Desalination* 247, 79-92.

root diameter and an increase in specific root length (SRL, length per unit weight of roots), significant only at the highest dosage, together with a tendency towards enhancement of translocation of heavy metals to the shoot – both positive traits for phytoremediation purposes.

Abbreviations: DAS: days after sowing; HA: humic acids; HM: heavy metals; RSS: root-to-shoot ratio; SRL: specific root length (length per unit weight of roots); SRR: shoot-to-root-ratio.

Introduction

Phytoextraction denotes the use of plants to remove heavy metals from soils, sediments or water into harvestable plant biomass, taking advantage of the ability of plants to absorb these pollutants through their youngest roots and translocate them to the shoots. When using phytoremediation to treat metal-polluted sites, two different strategies can be applied. One is to use hyperaccumulator plants, such as *Thlaspi caerulescens* or *Alyssum bertolonii* (Reeves and Brooks, 1983), which take up selected metals at high concentrations; the other is to cultivate fast-growing species able to uptake several metals but at low concentrations. In both cases, the ability of the root system to colonise metal-polluted soils is the key factor to phytoextraction efficiency. For a highly toxic metal like mercury, a strong relationship between root accumulation and root surface area or root length density was found (Cocking et al., 1995; Heeraman et al., 2001).

Knowledge about the plant mechanisms responsible for metal extraction and the factors affecting the bioavailability of various heavy metals in soil is still limited. Metal bioavailability is affected by such factors, as cation exchange capacity (CEC), soil pH, amount of fertilisers, and abundance of chelators. As all these may be modified, there is

great attention in applied research to improve phytoextraction through agricultural practices. For instance, chelators such as ethylene-diamine-tetra-acetic acid (EDTA) can increase the solubility of metal cations and their bioavailability for plants. However, apart from the positive effects of EDTA on metal phytoextraction, negative effects on soil have also been described. The non-selective extraction of metals can be detrimental for plant growth, because alkaline earth cations like Ca and Mg, which are essential for plant growth (Barona et al., 2001), can be solubilised. EDTA is also persistent in soil, may remain adsorbed to soil particles even after soil remediation (Wasay et al., 1998), and causes reduction of plant growth (Chen and Cutright, 2001).

New, more readily degradable amendments, such as EDDS (ethylene-diamine-disuccinic acid) and NTA (nitrilotriacetic acid), have recently been tested with good results (Wenger et al., 2003). Nevertheless, widespread natural molecules, like humic substances, may be used as alternatives to synthetic chelators. The term *humic substances* refers to a class of naturally occurring organic materials commonly found in soils, sediments and natural waters, which derive from the decomposition of plant and animal residues (MacCarthy, 2001). Humic acids (HA) represent the fraction of humic substances insoluble in water in acidic conditions, which becomes soluble and extractable at higher soil pH. Molecules of humic acids are characterised by acidic groups such as carboxyl and phenol OH functional groups (Hofrichter and Steinbüchel, 2001) and, when applied to the soil, play an important role in the transport, bioavailability and solubility of heavy metals (Lagier et al., 2000). It has been demonstrated that HA contribute to reducing the physical mobility (diffusion, mass flow) of various metal species (e.g., Cu, Pb, Zn, Ni) in soil, and thus reduce the consequent risk of lateral or vertical contamination of water bodies, as acetic acid extraction of metals is generally reduced with HA (Halim et al., 2003).

The use of humic substances in phytoremediation has not been explored so far, and little attention is devoted to it in current literature. Better uptake of molybdenum (Mo) in forage obtained in a polluted valley in Austria (meadow rendzina) was observed after application of large quantities (10 g kg^{-1} w/w, i.e., 1%) of humic acids, as a consequence of the increased extractable fraction of Mo (+28%) (Neunhäuserer et al., 2001). Increased concentrations of humic acids in soil, from 0.125 to 1.25 mg kg^{-1} , have also been found to improve the solubility of mercury in modified Hg-contaminated mine tailings, especially

when thiosulphate salts (ammonium and sodium) were added to the medium (Moreno et al., 2005). The same authors showed that translocation of Hg to the shoot varied significantly among plant species; however, the high translocation rate observed in *Brassica juncea* in the presence of ammonium thio-sulphate was suppressed when HA was also added to the nutrient solution, probably because of the retention of Hg-HA complexes at root level.

Improved phytoextraction efficiency of Cd was found by Evangelou et al. (2004) as a consequence of the application of high dosages of humic acids, through increased Cd bioavailability in soil. Addition of humic acids at a very high dose (20 g kg⁻¹ of soil) increased Cd concentration in the shoot from 30.9 to 39.9 mg kg⁻¹ in *Nicotiana tabacum*, but not at a dose of 10 g kg⁻¹ – probably due to soil acidification or the formation of Cd-humic acid complexes easily absorbed by plants.

The hormone-like effect of humic substances has recently been investigated in crops (Delfine et al., 2005) and forest species (Pizzeghello et al., 2000) – with contrasting results, depending on plant species, amount and method of application, soil pH, and interactions with soil microflora.

The effects of humic compounds on root growth and morphology have not been well documented so far, and there is a need to gain more insight on the relationship between root characteristics and HA application, especially for phytoremediation purposes.

In this study, the effects of applying humic and fulvic acids at various doses and methods of supply were evaluated in terms of both shoot and root growth in *Raphanus sativus* L. var. *oleiformis* Pers. cultivated in pyrite cinders severely polluted by various heavy metals. The ability to accumulate heavy metals in the shoot was also investigated, aiming at identifying related root morphological traits and proper agronomic practices for *in situ* phytoremediation. We used fodder radish, *R. sativus* var. *oleiformis*, because preliminary results (Vamerali et al., 2006) showed the best growth and removal capacities among various herbaceous crop species in this specific substrate.

Materials and methods

Experimental conditions

Fodder radish (*Raphanus sativus* L. var *oleiformis* Pers. cv. Siletta nova) was cultivated for 97 days in opaque PVC pots (0.5-m height, 57 mm inner diameter) filled with a mixture of pyrite cinders and siliceous fine sand (0.25÷0.1 mm USDA class diameter) (1/1 w/w), to reduce bulk density and soil penetration resistance, with five replicates. The cinders, remaining after sulphur extraction for sulphuric acid production (800 °C roasting temperature), were collected from a site of national interest called the “Lagoon of Grado and Marano and adjacent rivers” at Torviscosa (Udine, NE Italy, 45° 49’ 23” N, 13° 16’ 40” E, 3 m a.s.l.), previously described by Coletto et al. (2006). In the site, a horizon of cinders (about 1 m deep) was covered by a thick layer (about 0.2 m) of less polluted, carry-over, gravelly soil; beneath the cinders, a deep layer of impermeable clay ensured that metal leaching was almost negligible.

The cinders collected from the study site were characterised by high levels of five heavy metals (HM), i.e., Co, Cu, Pb, Zn and As, which exceeded the Italian limits fixed by D.M. 471/1999 for agricultural uses (Table 1). Uncultivated cinders, lacking in organic matter, were subalkaline (pH 7.3), with relatively high values of both bulk density (1.65) and electrical conductivity (EC=3.01 mS cm⁻¹).

Plants were watered at about 2-day intervals with 1:2 diluted Hoagland solution (about 50 mL per pot, to avoid percolation), in order to provide water and nutrients. Humic acids were applied at various concentrations (0.1 and 1 g kg⁻¹) and methods (mixed with cinders before sowing; applied on the top of substrate after sowing; foliar spraying) as listed in Table 2, in comparison with untreated controls (C). The dosages of humic acids were chosen on the basis of existing literature (Cooper et al., 1998; Moreno et al., 2005) and taking into account a reasonable cost for large-scale applications.

Table 1. Total contents of heavy metals in cinders in comparison with Italian maximum permitted concentrations (Decree of the Italian Ministry for Environment and Protection of the Territory No. 471/1999, agricultural uses) and their ratio.

Metal	Total content (mg kg ⁻¹)	Legal limit (mg kg ⁻¹)	HM content / limit
As	909	20	45 / 1
Co	103	20	5 / 1
Cu	1786	120	15 / 1
Pb	506	100	5 / 1
Zn	2473	150	16 / 1
Cd	0.47	2	1 / 4
Cr (total)	2.51	150	1 / 60
Mn	111	-	-
Ni	3.22	120	1 / 37

A commercial liquid formulation (Humic Super, Tiller, Verona, Italy), containing 12% of organic matter and 10.1% of humic and fulvic acids (i.e., 84% of organic matter), extracted from leonardite by means of an alkaline solution (KOH), was used as the source of these organic compounds. The formulation had pH 9.0, C/N 63 and 0.6% of organic nitrogen. Treatments 0.1 M and 1 M were obtained by adding respectively 0.1 and 1 g HA kg⁻¹ of soil (w/w) to distilled water (20% v/w) and homogenisation before filling the pots. Pots used for controls (C), top application (0.1 T) and foliar application (F) were filled with wet cinders (20% moisture) without HA. Just after sowing, for treatment 0.1 T, a dose of 0.1 g HA kg⁻¹ of soil was applied through irrigation (50 mL per pot). For foliar application, a solution 0.1 g HA L⁻¹ was sprayed once a week (dripping was avoided), for 3 weeks, starting from 51 days after sowing (DAS).

Table 2. Treatments with humic acids (HA) and their abbreviations.

Treatment	Dose and method of application of HA
C	Untreated controls
0.1 T	0.1 g kg ⁻¹ top application, at sowing
F	Foliar spraying (0.1 g L ⁻¹ , once a week for 3 weeks, starting from 51 DAS)
0.1 M	0.1 g kg ⁻¹ mixed with cinders
0.1 MF	0.1 g kg ⁻¹ mixed with cinders plus foliar application (as F)
1 M	1 g kg ⁻¹ mixed with cinders
1 MF	1 g kg ⁻¹ mixed with cinders plus foliar application (as F)

Growth analysis and detection of heavy metals

The dynamics of leaf expansion were determined by 6 observations at about 10-day intervals. The first measurement of leaf area was made at 51 DAS by drawing the leaf shapes on white paper, cutting them out, and measuring the area with a LI-3100 area meter (Li-Cor Inc, Lincoln, Nebraska, USA). Starting from the second observation (62 DAS), leaf area was estimated from length and width measurements according to the linear regression calculated at 51 DAS:

$$Y = 0.8452 X - 0.0113$$

where X is $\sum_{i=1}^n (width_i \times length_i)$ of n lobes in a leaf, and Y is the actual area of the same leaf.

The correlation was derived from measurements of maximum and minimum lengths of leaves or their lobes with the actual leaf area ($n=81$; $P \leq 0.01$; $R^2=0.96$).

Starting from 55 DAS, at about 10-day intervals, the content of chlorophyll was measured indirectly with a SPAD 502 chlorophyll meter (Konica-Minolta, Hong Kong) on the youngest fully-developed leaf, as an indicator of plant nutritional status (nitrogen) and possible damage due to heavy metals.

At the end of the experiment (97 DAS), when the plants had almost reached the flowering stage, they were harvested and shoot biomass (fresh weight) was measured. Dry mass was determined by oven-drying the shoots (105 °C, 24 hours).

Acid digestion was performed in a microwave oven (Milestone ETHOS 900, Bergamo, Italy): samples were prepared with about 0.4 g of dried biomass, and 5 mL of nitric acid (70% v/v) and 1.5 mL hydrogen peroxide (130 v/v) were added. Contents of HM were revealed by ICP-OES (Inductively Coupled Plasma – Optical Emission Spectroscopy) (Spectro Ciros Vision, Spectro Analytical Instruments, Kleve, Germany). In addition to the five above-limit heavy metals, the concentrations of Cd, Cr, Mn and Ni in shoot tissues were also measured.

Root profiles

At harvest, detailed root analysis was performed by subdividing the profiles into 70-mm deep intervals and storing the subsamples at -18 °C. Subsequently, on thawed samples, an oxalic acid solution (2% w/v) was added, in order to facilitate separation of soil particles from roots through a flotation system, thus collecting roots on a sieve (500-µm mesh), as described in Oliveira et al. (2000). Roots were stored in ethanol solution (12% v/v) at 4 °C. For scanning, roots were arranged on a transparent Plexiglas tray with a thin layer of water (around 3 mm), limiting overlaps between roots and possible extraneous objects for better accuracy. One-bit 375-DPI TIFF-format root images were acquired and analysed by KS300 Rel. 3.0 software (Carl Zeiss Vision GmbH, München, Germany). During analysis, only graphic objects having a P^2/A ratio (i.e., P =perimeter; A =area) higher than 75 and area greater than 2 pixels (to exclude pixel noise) were measured as roots, as previously suggested in Vemerali et al. (2003). Following the procedure proposed by those authors, the length of roots in a sample was calculated by means of the algorithm *FbLm* (Fibre Length modified), as follows:

$$FbLm = \frac{P + \sqrt{P^2 - 16A}}{4}$$

The mean diameter in a sample was calculated by dividing the area of roots by their length. Subsequently, roots were oven-dried (105 °C, 24 hours) for determination of dry biomass and their contents of heavy metals were measured, as described above.

Bioavailability of heavy metals

At the beginning of the experiment, the bioavailable fractions of Cd, Ni, Pb, Cu and Zn in uncultivated cinders were determined by the method of Lindsay and Norwell (1969). The bioavailable heavy metals were extracted from 50 g of dried soil through 100 mL of a solution of DTPA (diethylene-triamine-pentacetic acid) (1.97 g L⁻¹), calcium chloride bihydrate (1.46 g L⁻¹) and triethanolamine (14.92 g L⁻¹), at pH 7.3 under shaking (2 hours, 60 cycles/min). After a 5-minute centrifugation (5000 rpm), the supernatant was filtered on filter paper and analysed by ICP-OES.

Statistical analysis

Statistical analysis on shoot and root data was performed by ANOVA. The LSD test (Least Significant Difference) was used to test differences ($P \leq 0.05$) among treatments. Data were analysed with the CoStat 6.2 program (CoHort Software, Monterey, CA, USA).

Results and discussion

Shoot analysis

With respect to controls, application of small amounts of HA, regardless of method of application, had a slight, not significant, positive effect on shoot biomass, and thus the phytotoxicity of heavy metals on plant growth was partially mitigated. Instead, high doses of HA markedly reduced shoot weight (Fig. 1), probably because of the higher solubilisation of some heavy metals, which was preliminarily detected in uncultivated cinders for Cd, Cu and Ni (Table 3).

Table 3. Bioavailable fraction (% of total) of some heavy metals in uncultivated wastes (50%-diluted cinders, with sand) at 0.1 and 1 g HA kg⁻¹ of soil (0.1 M and 1 M) in comparison with controls (C) (analysis in duplicate).

Metal	Treatment		
	C	0.1 M	1 M
Cd	49.6	47.9	55.6
Cu	7.9	7.7	8.2
Ni	5.0	4.3	5.7
Pb	13.8	14.9	13.0
Zn	1.8	0.7	0.8

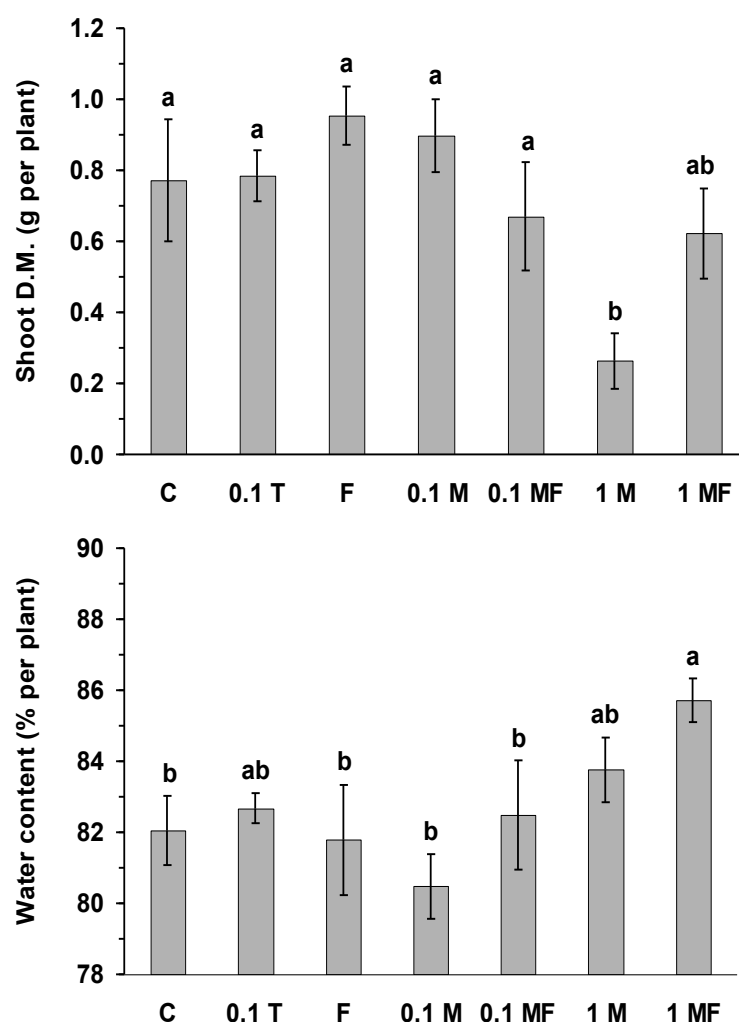


Figure 1 – Shoot dry matter (above) and humidity of fresh biomass (below) ($\pm$ S.E., $n=5$) in seven treatments. Vertical bars: standard error. Different letters indicate statistically significant differences (LSD test, $P \leq 0.05$).

In treatment 1 M, toxicity was very high, and two replicates perished before the end of the experiment. For high-dose HA treatments, decreases in shoot biomass of 66% and 19% were found for 1 M and 1 MF, respectively, suggesting that additional foliar application of humic acids tends to reduce phytotoxicity. In this regard, although foliar application alone did not have any significant effect on leaf growth, faster leaf expansion was found for treatments 1 MF and 0.1 MF, compared with 1 M and 0.1 M, as evidenced by the standardised values of leaf area (Fig. 2).

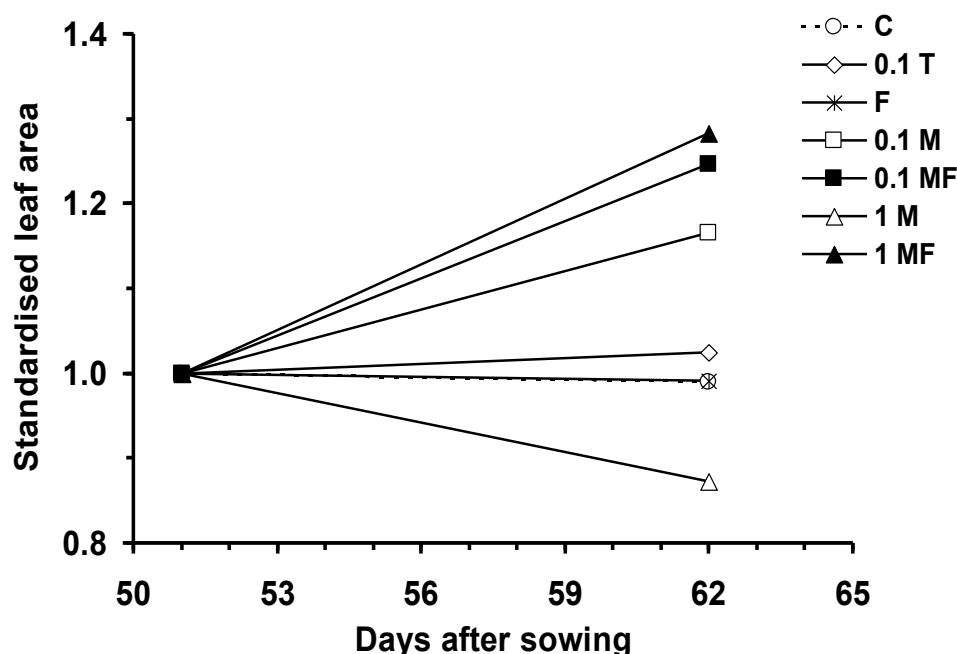


Figure 2 – Rate of leaf expansion (standardised on initial values) between first and second foliar application of HA (11 days of interval) in different treatments (n=5).

Shoot biomass was positively correlated with fresh weight ($R^2=0.88$), but high dosages of humic acids were unexpectedly associated with higher water contents in the whole plant (1 MF and 1 M vs. C: 86% and 84% vs. 82%) (Fig. 1). As elevated concentrations of heavy metals were also found in these treatments, together with a general positive correlation between total HM concentration and water content in shoot tissues ($P \leq 0.05$, $R^2=0.48$), these results contribute to support the hypothesis of the osmotic effect of HM. For radish, Dube et al. (2004) observed the reduction of both shoot growth and relative water contents of leaves as a consequence of applying high concentrations of Cr in a nutrient solution. Similar results were reported for *Brassica alboglabra* by Horng et al. (2002) in hydroponic culture solution: the higher the concentration of heavy metals in the roots, the higher the water stress. The only observation of the opposite trend in leaf water contents was found by Chatterjee and Chatterjee (2000) for cobalt in cauliflower, probably due to reductions in water potential and transpiration rate. Some authors (Lefevre et al., 2005), have also observed that plant responses may vary over time, as long exposure to heavy metals – in their case, cadmium – may improve the ability of *Zygophyllum fabago* to restore a more favourable water balance.

The positive effects of foliar application in reducing the phytotoxicity of high amounts of HA in soil was also observed in terms of SPAD values (Fig. 3). There was no interaction between ‘Treatment’ and ‘Observation date’; the lowest contents of chlorophyll and the worst nutritional status were found in treatment 1M. This suggests that HA affect nitrogen uptake and integrity of leaf pigments when higher concentrations of heavy metals are reached in shoots, as evidenced by Kupper at al. (2003) as a consequence of substitution of Mg by Cu in chlorophyll molecules.

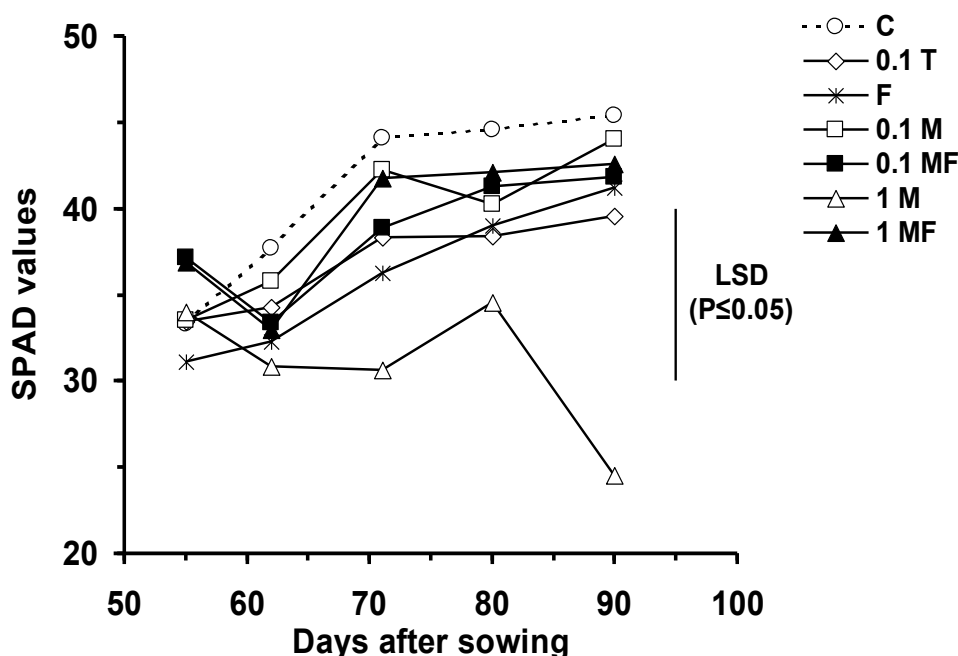


Figure 3 – Dynamics of SPAD (index of leaf chlorophyll content) during experiment (n=5). Vertical bar: LSD value ($P \leq 0.05$) for interaction ‘Treatment’ $\times$ ‘Time’: 10.01.

Uptake of heavy metals

At harvest, plants revealed various concentrations of heavy metals, depending on treatment and metal. As regards the five above-limit pollutants in cinders, no differences were found among treatments for Pb and As, but higher concentrations of Cu were generally found with application of HA, except foliar F, irrespective of dose (Table 4). Lastly, for Zn and Co, increased concentrations were induced by higher doses (1 MF for Zn) and top application (0.1 T for Co). Values higher than controls were also found for 1 M for some below-limit heavy metals, such as Mn and Cr (Table 4), and some nutrients like

phosphorus and boron (data not shown), confirming that appreciable amounts of metals/nutrients become more available in soil through the natural chelating effect of humic substances. For all heavy metals considered, concentrations in HA treatments were at least similar to controls but never lower. Among the most abundant heavy metals in cinders, variation among treatments was not very high, in general below 18%, except for Co (30%), with a minimum for Pb (12%), suggesting that the concentrations of these heavy metals in shoots may vary by relatively small quantities in a biomass plant like fodder radish, but the opposite was true for low-abundant metals (e.g., C.V.: Ni=77%, Cr=59%).

Table 4. Concentrations (mg kg⁻¹ D.M., n=5) of nine heavy metals in shoot and root tissues. For each metal, means and coefficients of variation (CV %) of treatments are listed. Within same metal and plant organ, different letters refer to statistically significant differences among treatments (LSD test, P≤0.05), n.s. absence of significance.

	C	0.1 T	F	0.1 M	0.1 MF	1 M	1 MF	Mean CV (%)	
<i>Shoot</i>									
As	6.4 n.s.	7.2	5.8	5.2	6.2	5.2	4.6	5.8	15
Co	0.08 b	0.18 a	0.10 b	0.10 b	0.09 b	0.12 ab	0.09 b	0.11	30
Pb	1.3 n.s.	1.5	1.3	1.3	1.4	-	1.1	1.3	12
Cu	73 c	112 a	73 c	86 abc	84 bc	111 ab	85 abc	89	18
Zn	85 b	89 ab	80 b	74 b	78 b	101 ab	116 a	89	16
Ni	1.1 n.s.	1.1	0.7	5.1	2.1	2.2	1.2	1.9	77
Cr	0.47 b	0.48 b	0.25 b	0.34 b	0.26 b	1.06 a	0.43 b	0.47	59
Cd	0.44 ab	0.57 a	0.30 ab	0.42 ab	0.22 b	0.63 a	0.56 a	0.45	34
Mn	44 c	65 abc	48 bc	53 abc	54 abc	79 a	69 ab	59	21
<i>Roots</i>									
As	170 n.s.	162	172	155	164	136	139	157	9
Co	13.6 ab	12.3 b	11.6 b	11.1 b	10.4 b	17.5 a	12.7 b	12.7	18
Pb	576 b	378 bc	541 b	229 c	368 bc	969 a	561 b	518	46
Cu	845 cd	1026 abc	1012 bcd	768 d	846 cd	1163 ab	1270 a	990	18
Zn	1211 a	1081 ab	1015 abc	892 bc	978 abc	802 c	782 c	966	16
Ni	3.8 b	5.1 b	5.1 b	5.2 b	5.6 b	11.4 a	5.7 b	6.0	41
Cr	7.5 ab	3.4 bc	8.0 abc	1.6 c	3.9 bc	16.9 a	4.4 abc	6.5	78
Cd	-	-	-	-	-	-	-	-	-
Mn	51 c	73 bc	53 c	129 b	113 bc	275 a	253 a	135	68

As the cinders of the polluted site were pluricontaminated, an attempt was made to provide an overall evaluation of all the heavy metals accumulating in the harvestable biomass by summing the standardised concentrations of nine heavy metals, including both

above- and some below-limit pollutants (i.e., As, Co, Pb, Cu, Zn, Ni, Cr, Cd, Mn). Standardisation was obtained by dividing the concentration by a threshold value for hyper-accumulation found in the literature for each pollutant in vascular plants (Terry and Bañuelos, 2000). The thresholds were 100 mg kg⁻¹ D.M. for Cd, 1,000 mg kg⁻¹ D.M. for As, Co, Pb, Cu and Ni, and 10000 mg kg⁻¹ D.M. for Zn, Mn and Cr. The resulting cumulative accumulation capability was generally higher in treated plants than in controls – apart from foliar application F – with the highest values at high dosage of HA (1 M) and for top application (0.1 T) (Fig. 4).

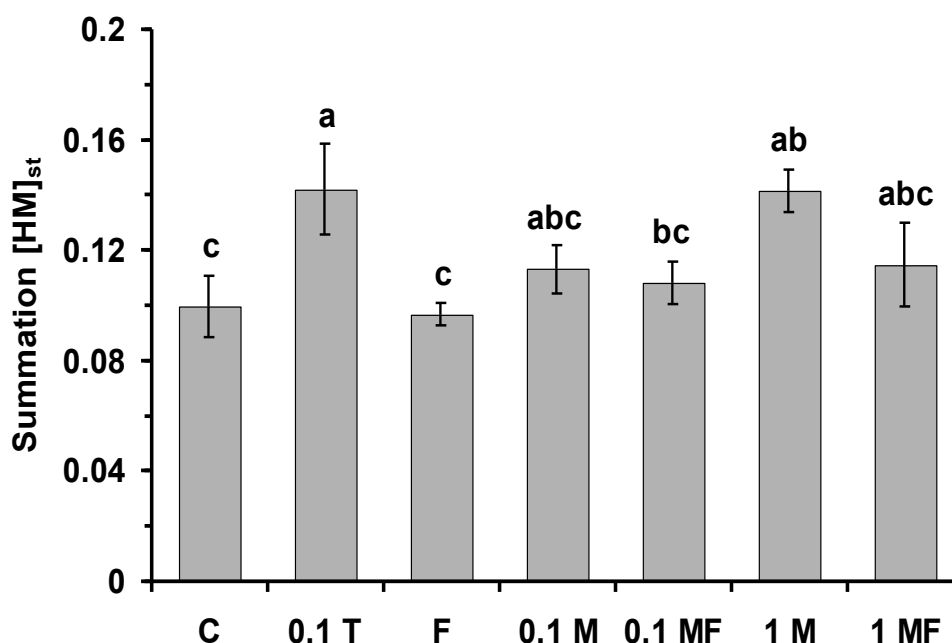


Figure 4 – Comparison of sums of standardised concentrations ($\pm$ S.E., $n=5$) of analysed heavy metals in shoots: As, Co, Pb, Cu, Zn (above legal limit) and Ni, Cr, Cd, Mn (below legal limit). Different letters indicate statistically significant differences (LSD test, $P \leq 0.05$).

Concentrations of various HM in roots (whole system) were several times higher than those of the shoots – an order of magnitude of some hundreds for Co and Pb, and some tens for Cu and Zn (Fig. 5). Application of HA led to contrasting results in terms of concentrations of different heavy metals, values generally being lower than controls for Zn and Pb, higher for Cu, and similar for As and Co (Table 4). Variations (CV) among treatments were similar to those of the above-ground biomass for Cu and Zn, and higher for

Pb, but lower for Co and As, suggesting that an improvement of translocation is possible, at least for the most variable HM. In this regard, with few exceptions, HA allowed an increase in metal translocation to the shoot, although in some cases not significantly – as evidenced by the lower ratio of concentrations between root and shoot compared with controls for Co, Pb, Cu and Zn, but not for As, the latter showing similar values among treatments (Fig. 5).

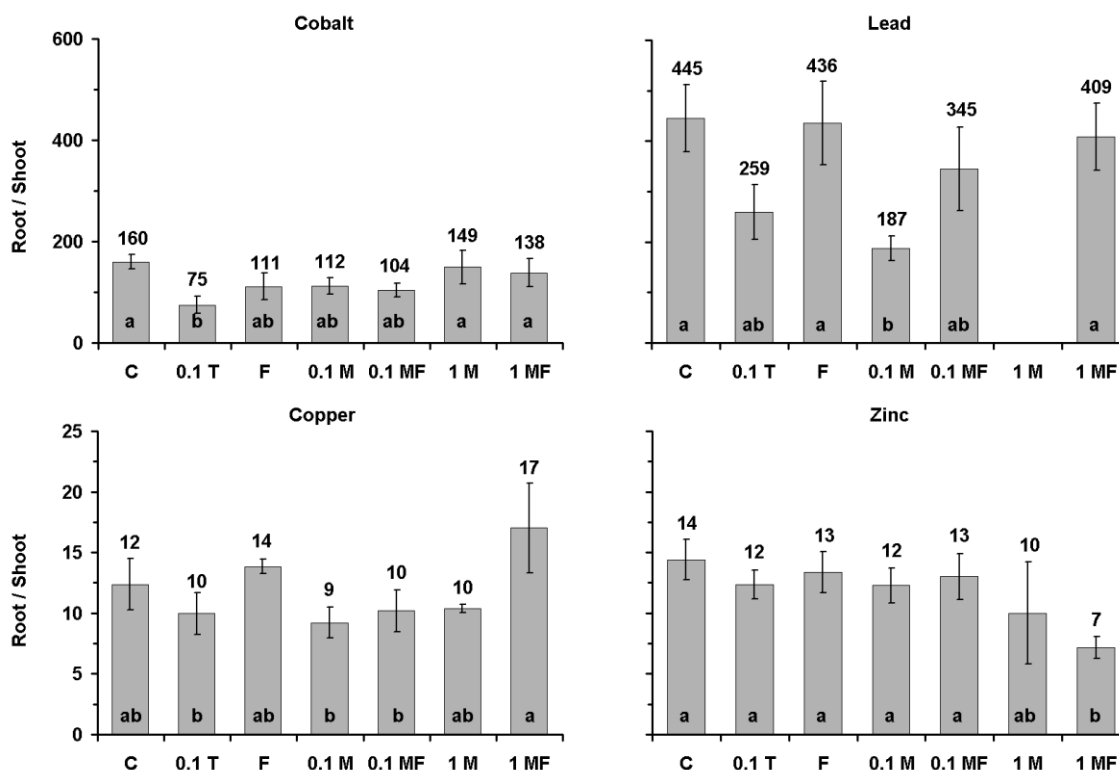


Figure 5 – Root-to-shoot concentration ratio ($\pm$ S.E., $n=5$) of Co, Pb, Cu, and Zn (values are reported above bars). Different letters indicate statistically significant differences (LDS test, $P \leq 0.05$).

With the simultaneous presence of competing inorganic and organic chelants (ammonium and sodium thiosulphate vs. humic acids), some authors, e.g., Moreno et al. (2005), have hypothesised the prevalent retention of Hg-HA complexes at root level, with reduced translocation to shoots. Instead, in our experiment, dealing with various heavy metals, HA exerted a generally positive effect. Maximum translocation was found for Zn at 1 M and 1 MF (i.e., high dosages of HA) and for Pb at 0.1 M, 0.1 MF and 0.1 T (i.e., low dosages of HA); foliar application alone did not modify the translocation of any heavy metals to the shoot. For Pb, which is not an essential metal for plants, root retention was

particularly high compared with the other metals, as also found by other authors (e.g., Abd El Hamed et al., 2001; Saygideger and Dogan, 2005).

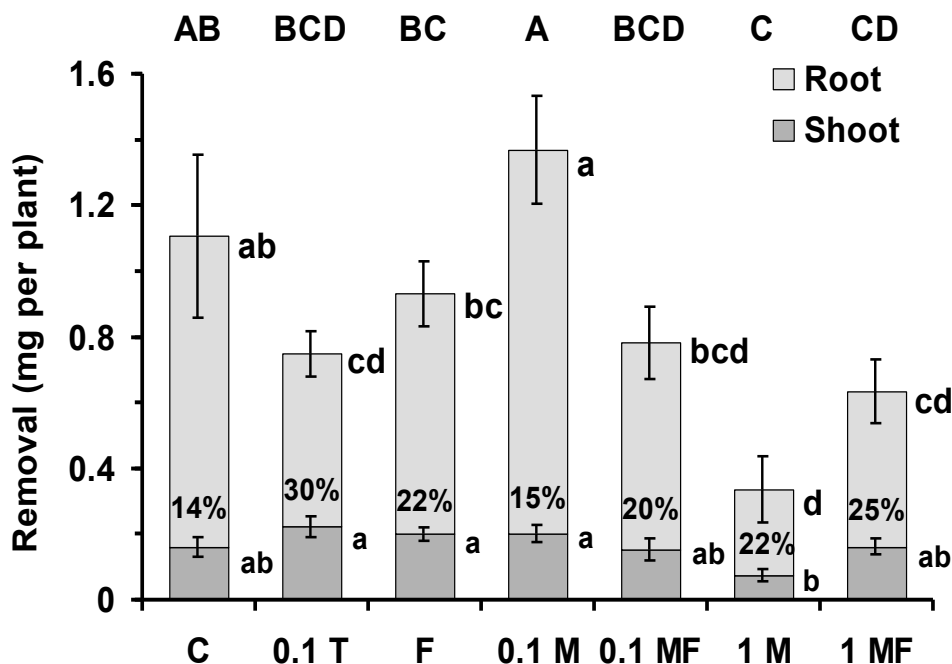


Figure 6 – Shoot removal ($\pm$ S.E., $n=5$) of nine heavy metals: As, Co, Pb, Cu, Zn, Ni, Cr, Cd, Mn. Different letters indicate statistically significant differences (LSD test, $P \leq 0.05$). Small letters: shoot or root; capital letters: overall removal (shoot + root). Percentages refer to shoots.

Removal of heavy metals by above-ground biomass, calculated as product between dry weight and pollutant concentrations, was higher when shoot biomass reached higher values, due to their significant correlation (coefficient of correlation $r=+0.89$, $P \leq 0.05$), whereas the incidence of concentrations of HM was much less important ($r=+0.13$, n.s.). Therefore, for almost all the metals considered here, a tendency towards higher quantities was found for treatment 0.1 T, followed by 0.1 M and F (Fig. 6), almost following the same hierarchy as shoot biomass. The maximum overall shoot removal (nine HM) was in 0.1 T (0.22 mg per plant), and minimum in 1 M (0.08 mg per plant), mainly represented by three heavy metals, i.e., Zn (35% of removal, average of treatments), Cu (36%) and Mn (24%). The high concentration of HM at root level indicates that, in these specific conditions, with respect to the total amount absorbed by the plant, the contribution of the shoot in removing

pollutants is moderate (21% on average) (Fig. 6), especially when compared with the higher values (around 80%) found in mature plants of fodder radish cultivated in a agricultural pluricontaminated sandy-loamy sub-acidic soil rich in organic matter (pot experiment) (Mosca et al., 2004; Vamerali et al., 2004). Considering both root and shoot biomass, the total amounts of various heavy metals reached the highest value in 0.1 M (1.37 mg per plant) and the minimum in 1 M (0.34 mg per plant).

Root characteristics

Table 5. Root characteristics (values per plant $\pm$ S.E., n=5) in seven treatments at end of experiment. Within same parameter, different letters refer to statistically significant differences (LSD test, $P \leq 0.05$).

Treatments	Weight (g D.M.)	Length total (m)	Length top 7 cm (% of total)	Diameter (μm)	SRL (m g^{-1})	SRR (g g^{-1})
C	0.334 $\pm$ 0.08 b	82 $\pm$ 22 b	53 $\pm$ 5 ab	148 $\pm$ 7 a	258 $\pm$ 46 c	2.7 $\pm$ 0.7 ab
0.1 T	0.195 $\pm$ 0.03 bcd	56 $\pm$ 10 bc	67 $\pm$ 8 a	134 $\pm$ 7 abc	286 $\pm$ 22 bc	4.8 $\pm$ 1.4 a
F	0.257 $\pm$ 0.03 bc	75 $\pm$ 11 b	30 $\pm$ 9 c	126 $\pm$ 4 c	293 $\pm$ 25 bc	3.9 $\pm$ 0.6 ab
0.1 M	0.541 $\pm$ 0.09 a	120 $\pm$ 4 a	48 $\pm$ 3 bc	143 $\pm$ 3 ab	241 $\pm$ 31 c	1.8 $\pm$ 0.3 b
0.1 MF	0.269 $\pm$ 0.07 bc	73 $\pm$ 7 b	64 $\pm$ 4 ab	139 $\pm$ 6 abc	309 $\pm$ 41 bc	2.5 $\pm$ 0.2 ab
1 M	0.047 $\pm$ 0.02 d	28 $\pm$ 6 c	55 $\pm$ 2 ab	129 $\pm$ 6 bc	432 $\pm$ 77 a	3.7 $\pm$ 0.2 ab
1 MF	0.161 $\pm$ 0.04 cd	60 $\pm$ 13 c	46 $\pm$ 5 abc	131 $\pm$ 4 bc	386 $\pm$ 30 ab	4.8 $\pm$ 1.3 a

Application of humic acids resulted in marked modification of root morphology, as described by parameters like weight and length, diameter, specific root length (SRL, length per unit weight of roots) and relationship with above-ground biomass. As expected, HA were more phytotoxic at root level than in the shoot, probably because of their chelating effect. According to this hypothesis, except for treatment 0.1 M only, which enhanced root length and biomass (+46% and +62%, respectively), and foliar – having no significant effect – a noticeable reduction in root growth was detected for the other HA treatments (Table 5). Maximum reduction was found for high dosages of HA, considerable in both weight (1 M: -86%, 1 MF: -52%, vs. controls) and length (1 M: -66%; 1 MF: -27%). For top application of low concentrations of HA (0.1 T), reduction in root growth was less evident, probably due to the gradual deepening of HA from the shallow top layer. In all

cases, variations in root weight were more marked than in root length, as a general reduction in root diameter (-10% on average) together with an increase in specific root length were imputed to HA. For SRL, values ranged between 258 m g⁻¹ D.M. of controls to 386 and 432 m g⁻¹ of 1 MF and 1 M, respectively – an increase to be considered positively for better efficiency in metal uptake. As observed in preliminary trials, root diameters of fodder radish at flowering were higher in the polluted site of Torviscosa (Udine) than in the silty-loamy soil of the Experimental Farm of the University of Padova (Legnaro, Italy) (179 µm vs. 137 µm, data not published), due to the higher bulk density of the pyrite cinders. The addition of sand to the cinders and the application of HA led to a favourable reduction in diameter, down to values almost similar to those found in the normal agricultural conditions of Legnaro. The coefficients of variation of root diameter along the profiles were very low (range: 8% to 19%), the minimum being found in 0.1 M and maximum in 0.1 T. For the latter, as increased root diameters were found with increasing depth, and application of HA was generally associated with reduced diameter, the previous hypothesis of gradual deepening of HA into the cinders was further supported. In this regard, as the phytotoxicity of the cinders was also evidenced by the high fraction of root length confined to the top 70-mm layer, maximum values was again found in the case of top application of HA (67%) and minimum for foliar treatment (30%) (Table 5).

As regards the shoot-to-root ratio (SRR), a trend towards higher values was associated with application of HA, but not at low concentrations (0.1 M, 0.1 MF). Although for phytoremediation purposes an optimal SRR should be reached and this may be difficult to identify, in our experiment the best uptake of heavy metals (0.1 M, whole plant) was associated with the lowest SRR. This fact suggests the important role of root growth in directly improving metal uptake, but also indirectly through soil acidification (metal mobility). Compared with controls, some treatments with low dosages of HA (i.e., F, 0.1 M), reduced soil pH, whereas at high ones soil pH was more similar to the initial value 7.3 (uncultivated cinders without HA) (Fig. 7). As the effect of HA application on soil pH was almost negligible in uncultivated cinders (pH 6.8 and 6.9, for 0.1 M and 1 M, respectively), this result was mainly attributed to the different extent of root growth and release of exudates, according to the significant negative correlation found between soil pH and root biomass ($R^2=0.22$). Further confirmation of this relationship derives from controls, in

which cultivation allowed soil pH to be reduced from 7.3 to 5.5. It is well-known that most heavy metals increase their mobility at low pH, with optimal values for each pollutant (Hornburg and Brümmer, 1993).

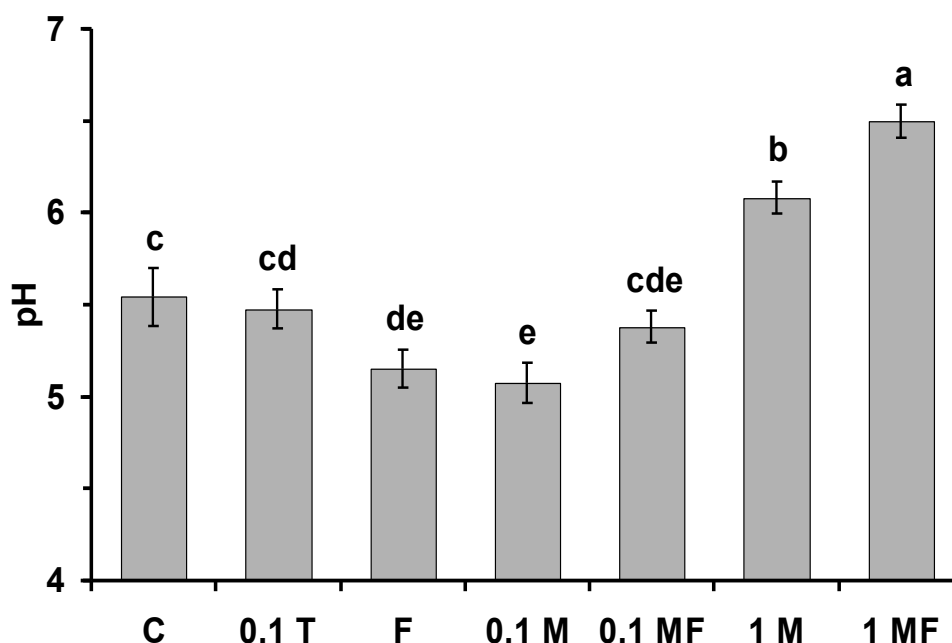


Figure 7 – Soil pH (average of profile, $\pm$ S.E., $n=5$) in seven treatments. Different letters indicate statistically significant differences among treatments (LSD test, $P \leq 0.05$).

Within the range of pH in this experiment (5-6.5), significant negative correlations ($P \leq 0.05$) with pH were found for shoot removal of As ($R^2=0.15$), Cu ($R^2=0.10$) and Pb ($R^2=0.14$), but not for Co and Zn, although the correlations were negative.

Within the variability of the experiment, the absence of correlation between root parameters and concentrations of heavy metals in shoots was expected for fodder radish, as a biomass species. In particular, as no correlation was verified between metal shoot concentration and the root-to-shoot ratio (RSR), the reciprocal of SRR, which is an index of abundance of roots per unit weight of shoot, the importance of root age in taking up heavy metals and the coefficient of translocation to shoots should be emphasised. Despite this, both RSR and root length were closely correlated with the total amounts of the nine heavy metals taken up by the plant (shoot + root) (Fig. 8), further evidence that should guide the use of low amounts of humic acids mixed with contaminated cinders before sowing.

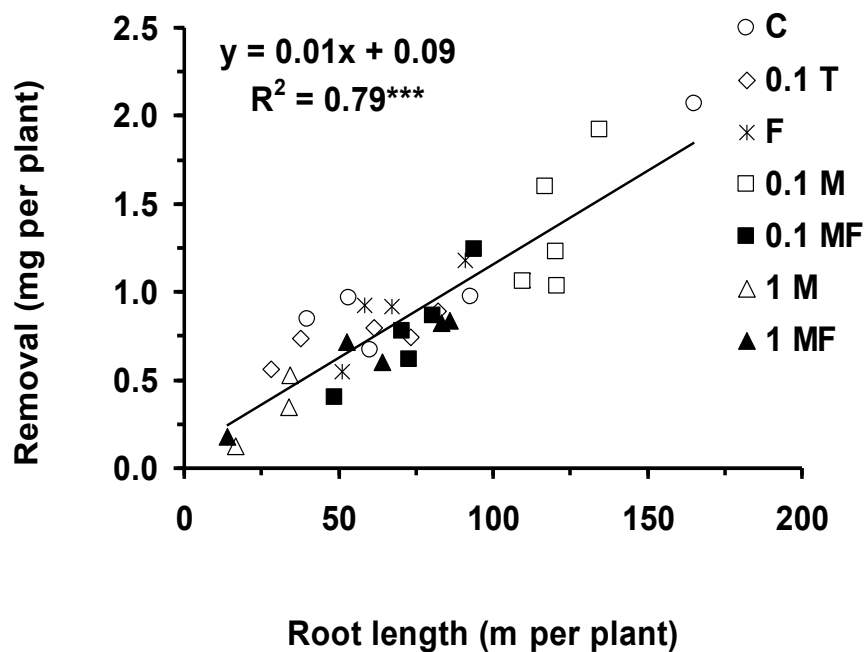


Figure 8 – Linear regression (n=35) between root length and plant overall removal (nine heavy metals, shoot + root).

Conclusions

The use of biomass plants for phytoremediation is of great interest, as their seeds are easily available and there is extensive information on their cultivation with respect to hyperaccumulators, but there is an urgent need to improve remediation efficiency through agronomic practices.

Although data refer to relatively early growth stages in pots, this experiment provides useful information for in situ cultivation of fodder radish, as preliminary results showed the

best growth and removal capacities among various herbaceous crop species (i.e., sunflower, alfalfa, Italian ryegrass, barley) in metal-polluted pyrite cinders.

In fodder radish, application of low doses of humic acids had a slight growth-promoting effect on the shoot, whereas high doses were phytotoxic, although with partial mitigation of these negative effects by additional foliar application. Due to their chelating effect, HA reduced root growth and diameter, and increased SRL, especially at high doses. Among tested treatments, small amounts of HA (0.1 g kg^{-1}) increased the amount of heavy metals taken up by the plant and their translocation to the shoot, with major benefits for Cu and Pb, thus suggesting their potential for use in phytoremediation. The greater root growth associated with this treatment, with respect to controls, may also allow plants to withstand periods of drought better when applied to our particular polluted site, in which fodder radish showed a maximum root depth of 0.3 m (Vamerali et al., 2006).

For practical applications, it may be possible to apply HA by spraying on the surface and promptly incorporating them into cinders by harrowing, but the cost of treatment should be carefully considered, as a 0.15-m deep interval, added at a dosage of 0.1 g kg^{-1} , requires more than 200 kg of HA per hectare.

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Chapter V. Effects of exogenous indolebutyric acid application to fodder radish (*Raphanus sativus* L. var. *oleiformis*) for phytoremediation of metal-polluted wastes

Abstract

The use of plant growth-promoting substances, like auxins, may potentially improve the phytoremediation of trace elements, as more roots are available for their uptake. In this view, a greenhouse pot experiment involved cultivating fodder radish (*Raphanus sativus* L. var. *oleiformis* Pers.) for about 3 months in As-Co-Cu-Pb-Zn-polluted pyrite wastes and treating the plants and/or substrate with indolebutyric acid (IBA).

IBA was given at different doses and ways of application, starting from 28 days after sowing (5 applications at 10-day intervals). Treatments were: foliar spraying (called F, 10 mg IBA L⁻¹), 0.1 and 1 mg IBA kg⁻¹ of wastes applied through irrigation (called 0.1ppm and 1ppm, respectively), and the same doses in association with foliar application (0.1ppm+F, 1ppm+F), in comparison with untreated controls (C).

With the exception of foliar spraying, IBA unexpectedly reduced the above-ground biomass at harvest – the higher the dose, the lower the growth. Roots did not follow a similar trend, and foliar treatment produced higher length with respect to controls (+16%). Concentrations of trace metals were generally enhanced by IBA in both roots and shoots, probably due to increased H⁺-ATPase activity, particularly for Co and Pb at the highest auxin dosage. Impaired above-ground yield caused by hormonal application was responsible for reduced mass removal of metals, and increases in water percolation and metal leaching.

These results reveal the detrimental effects of IBA in phytoremediation, at least within the concentrations tested, but the promising effects on rooting in foliar applications should be examined more thoroughly in order to identify more profitable dosages.

Abbreviations: DAS: days after sowing; EC: electrical capacitance; EDTA: ethylene-diamine-tetraacetic acid; IAA: indoleacetic acid; IBA: indolebutyric acid; ICP-OES: Inductively Coupled Plasma – Optical Emission Spectroscopy; NAA: naphthaleneacetic acid; TF: translocation factor.

Introduction

The use of plant growth-promoting substances may potentially improve the phytoremediation of trace elements, since they positively increase both shoot biomass and root extension, allowing greater acquisition of metals. Among hormones affecting plant growth, auxins play a major role, being involved in cell division, growth, maturation, organ differentiation, and several physiological processes (Trewavas, 2000). Auxins are also directly involved in cation uptake, having plasma membrane H^+ -ATPase as their final target (Hager, 2003). H^+ -ATPase acidifies the apoplast, whereas the cytoplasm becomes alkalised (Tode and Lüthen, 2001). Acidification of the apoplast weakens the cell wall, and the electrochemical gradient created across the plasma membrane leads to the opening of cation channels or activates membrane ion transport proteins, which results in an influx of cations (Liphadzi et al., 2006).

Very few studies regarding the use of auxins in phytoremediation are available in the literature. Among these, Lopez et al. (2005) found increased Pb concentrations in roots (+40%) and leaves (28×) of *Medicago sativa* L. when the plants received 100 μ M of indol-3-acetic acid (IAA) in addition to EDTA in the hydroponic solution. Liphadzi et al. (2006)

also found improvements in metal uptake in *Helianthus annuus* L.. These authors applied doses of 17 and 34 μM IAA per pot 3 times, using 3 and 6 mg L^{-1} of solution, respectively, equally divided between leaves and soil. In soil not amended with EDTA chelator, they observed increases in root growth which were associated with higher leaf Mn and Ni contents. In the results of Fuentes et al. (2000) on *Zea mays* L. sprayed with indolebutyric acid (IBA) and naphthylacetic acid (NAA), increases of 41% of Pb with IBA and 127% of Pb and 60% of Zn with NAA were observed, together with biomass reduction and high plant mortality.

Plant growth can also be enhanced through bacterially produced phytohormones. For instance, Sheng and Xia (2006) found increased growth of both root and shoot and Cd accumulation in *Brassica napus* L. as a result of soil inoculation with Cd-resistant IAA-producing bacteria. In the experiment of Dimkpa et al. (2008), production of siderophores by various *Streptomyces* strains was found to promote auxin synthesis via chelation of metals such as Cd, Cu and Ni, having the potential to inhibit synthesis of this hormone.

Within this framework, there is ample scope for testing the exogenous application of auxins. This possibility has rarely been studied in the literature, and indolebutyric acid (IBA), which is considered one of the most powerful root promoting hormones, is not tested in phytoremediation.

In this study, IBA was applied to *Raphanus sativus* L. var. *oleiformis* Pers. at various doses and ways of application, in order to verify possible increases in above- and below-ground growth and metal uptake. The experiment aimed at improving phytoextraction of severely contaminated pyrite wastes, for which radish has already shown a good degree of adaptation in preliminary experiments (Vamerali et al., 2006).

Materials and methods

Pot experiment set-up

Fodder radish (*Raphanus sativus* L. cv. Siletta nova) was cultivated for 75 days in cylindrical pots (490 mm high, 57 mm inner diameter) filled with metal-polluted pyrite cinders mixed with sand (1:1 w/w) to improve water drainage. The cinders, collected from a site of national interest called “Lagoon of Grado and Marano and adjacent rivers” (Udine, NE Italy), were produced by pyrite ore roasting for sulphur extraction and were severely contaminated by As, Co, Cu, Pb and Zn (Table 1), although the polluted site seldom contained Cd exceeding the Italian Guidelines Values (IVG) for agricultural uses (Ministerial Decree 152/2006).

Table 1. Concentrations for above- and some below-limit metals in pyrite wastes, in comparison with Italian Guidelines Values (IGV) for agricultural uses of soil (Ministerial Decree 152/2006).

Metal	Total content (mg kg ⁻¹) (A)	Legal limit (mg kg ⁻¹) (B)	(A)/(B)	DTPA- extractable (mg kg ⁻¹)
As	886	20	44/1	-
Zn	2,404	150	16/1	44.6
Cu	1735	120	14/1	142
Co	100	20	5/1	-
Pb	493	100	5/1	69.8
Cd	0.46	2	1/4	0.23
Ni	3.1	120	1/38	0.16
Cr (total)	2.5	150	1/61	-
Mn	108	-	-	-

The bottom of the pots were connected with black tubes to opaque PVC bottles (1 L) in order to collect leachate. One plant per pot was sown at the end of May and watered about twice a week with 1:2 diluted Hoagland solution. Pots were kept in greenhouses

throughout the experiment, with maximum and minimum temperatures of 26 °C and 18 °C, respectively.

Indole-3-butyric acid (IBA) was applied five times at ten-day intervals, starting from 28 days after sowing (DAS). Treatments (5 replicates) were: foliar spraying of IBA water solution (called F, 10 mg IBA L⁻¹, i.e., 50 µM), 0.1 and 1 mg (i.e., 0.5 and 5 µmol) IBA kg⁻¹ of soil applied through irrigation (in 100 mL of distilled water per pot) (called 0.1ppm and 1ppm, respectively). Two further treatments involved combining soil with foliar applications (called 0.1ppm+F and 1ppm+F). At the moment of IBA soil application, the untreated reference controls (C) were watered with equivalent amounts of distilled water. In foliar application, IBA solution was sprayed until the dripping point. Throughout the cycle, each pot received a total amount of 3.8 L of water.

Shoot measurements

During the growth period, leaf expansion was monitored at weekly intervals, starting on 11 July (47 DAS). Values of leaf area were obtained by non-destructive measurement of leaf diameters during the cycle, and by means of a LI-3100 leaf area meter (Li-Cor Inc., Lincoln, Nebraska, USA) at harvest. Final shoot biomass was measured after oven-drying at 105 °C for 24 hours, and metal contents were revealed by ICP-OES (Inductively Coupled Plasma – Optical Emission Spectroscopy).

Root investigation

Root electrical capacitance (EC) was recorded during the cycle at the same time intervals as leaf measurements on a hand-held digital ELC-131D electrical capacitance meter (Escort Instruments Corporation, Taipei, Taiwan) (Preston et al., 2004). After preliminary calibration, the following settings were adopted:

a) positively-charged electrode (stainless steel rod, 2 mm diameter) 0.55 m long, 0.3 m inserted into the potting soil (vertical), and cable connection 60 mm above-ground level, earthed 20 mm away from the stem base;

b) negatively-charged electrode: fine stainless steel needle, orthogonally inserted into the stem at a height of 10 mm (1 mm penetration into the stem).

Capacitance values (pF) were recorded at 1 kHz frequency, 24 h after watering (200 mL Hoagland solution per pot), to reach approximate field capacity and to allow roots to come into contact with the soil solution.

At harvest, roots were separated from soil through flotation by means of a hydraulic centrifugation device and collected in a 500-µm mesh sieve, as described by Oliveira et al. (2000). Separation of soil particles was facilitated by the addition of oxalic acid solution (2% w/v) in a beaker. Roots were stored at 4° C in an ethanol solution (10% v/v) until analysis. One-bit 400-DPI TIFF-format images of roots were acquired by digital scanning on a flatbed scanner (EPSON Expression 10000XL, Canada). Root length was measured by KS 300 image analysis software (Carl Zeiss Vision GmbH, München, Germany) following the procedure of Vamerali et al. (2003). Discrimination of roots against extraneous objects was performed according to an Elongation Index value (i.e., $\text{perimeter}^2/\text{area}$) greater than 60 and a minimum object area greater than 25 pixels. Root length (*FbL*) was derived from perimeter (P) and area (A) of the digital objects, as follows:

$$FbL = \frac{P\sqrt{P/A}}{4}$$

Subsequently, root dry mass and its metal contents were determined as for shoots.

Percolation water

Volumes of percolation water, collected throughout the experiment, were measured at harvest. ICP-OES analysis was used to calculate the extent of metal leaching.

In both plant tissues and water, besides the 5 above-limit metals, concentrations of Cd, Cr, Mn, and Ni were also revealed.

Statistical analysis

Statistical analysis was carried out by Statgraphics software (Manugistic Inc., Rockville, MD, USA). ANOVA and the LSD test ($P \leq 0.05$) were used to evaluate differences among means for growth parameters and metal concentrations.

Stepwise regression ($P \leq 0.05$), with both forward and backward variable selection, was applied to overall metal leaching in relation to 7 independent variables, i.e., shoot dry weight, leaf area (integral over time), root dry weight, root length, root electrical capacitance (integral over time) and metal concentrations in both shoots and roots.

The overall translocation factor (i.e., shoot-to-root metal concentration ratio) of 9 metals was also related to growth parameters (i.e., shoot dry weight, integral over time of leaf area and root electrical capacitance, root weight and root length) by multiple regression.

Before analysis, the data of each variable were standardised by subtracting the mean and dividing by standard deviation, so that the regression coefficients of variables in the model were comparable.

Results

Growth parameters

With the exception of foliar spraying, IBA unexpectedly reduced the whole plant biomass - the higher the dose, the lower the growth. At above-ground level, reduction was observed in terms of both weight and leaf expansion (Fig. 1), with a maximum decrement for the 1ppm treatment (-71% and -63% vs. controls, respectively). Root weight was also

generally reduced by IBA, the average drop from controls being -57% (Fig. 2). Root length did not follow the same trend as root weight, and foliar treatment showed higher values with respect to controls (+16%). For the other treatments, length was reduced by a minimum of 5% (0.1ppm) and a maximum of 27% (1ppm+F).

The fraction of roots colonising the top 7-cm layer of the pot waste reached about 52% of total length in IBA treatments compared with 44% of controls (Fig. 3). Specific root length (i.e., length-to-weight ratio) increased with IBA applications, with a maximum increment for foliar treatment (+109%). Root electrical capacitance (integral over time) was better correlated with root weight at harvest ($R^2=0.30$, $P\leq 0.01$) than with root length ($R^2=0.25$, $P\leq 0.01$). The dynamics of root electrical capacitance showed that differences among treatments emerged only in the last part of the experiment (i.e., from the beginning of August) (Fig. 4).

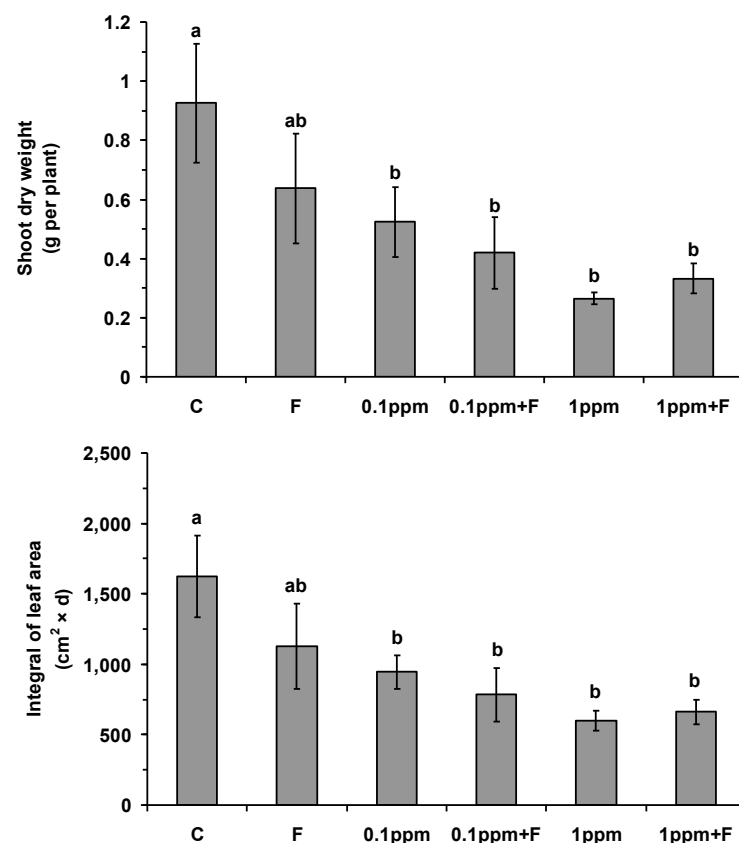


Figure 1. Shoot biomass at harvest (above) and integral over time of leaf area curves (below) ($\pm$ S.E., $n=5$) in various IBA treatments. Letters: differences among treatments (LSD test, $P\leq 0.05$).

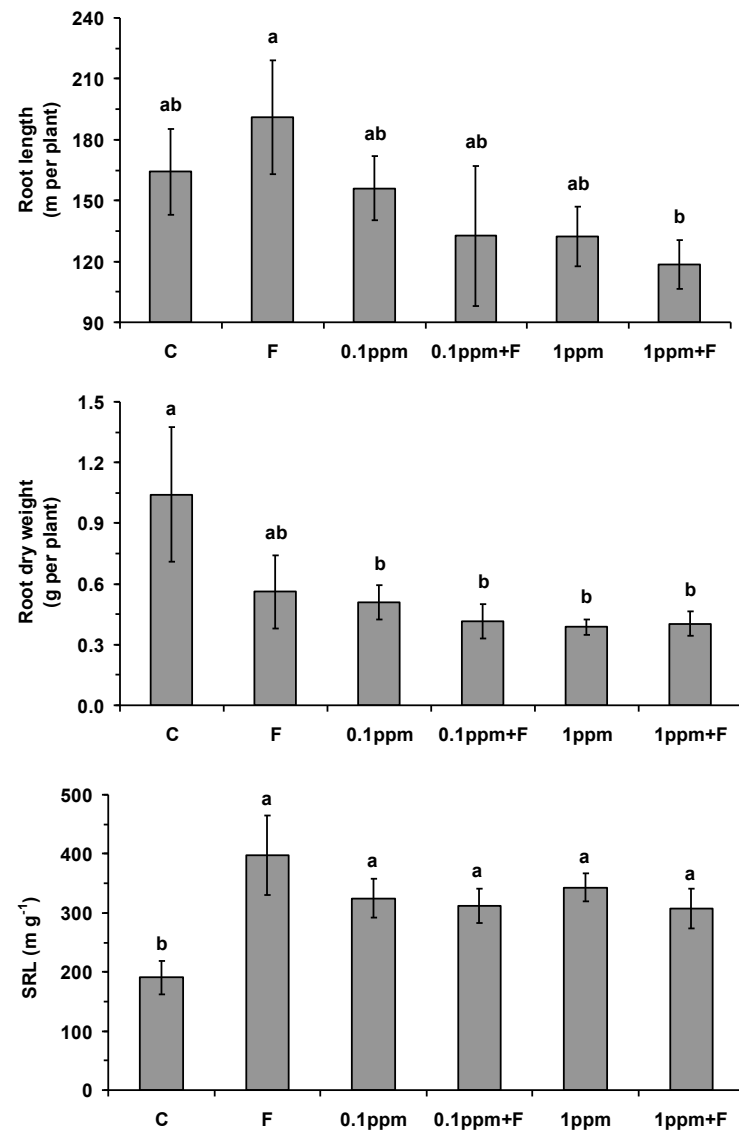


Figure 2. Root length (above) and biomass (middle) and specific root length (SRL) (below) at harvest ($\pm$ S.E., $n=5$) in various IBA treatments. Letters: differences among treatments (LSD test, $P \leq 0.05$).

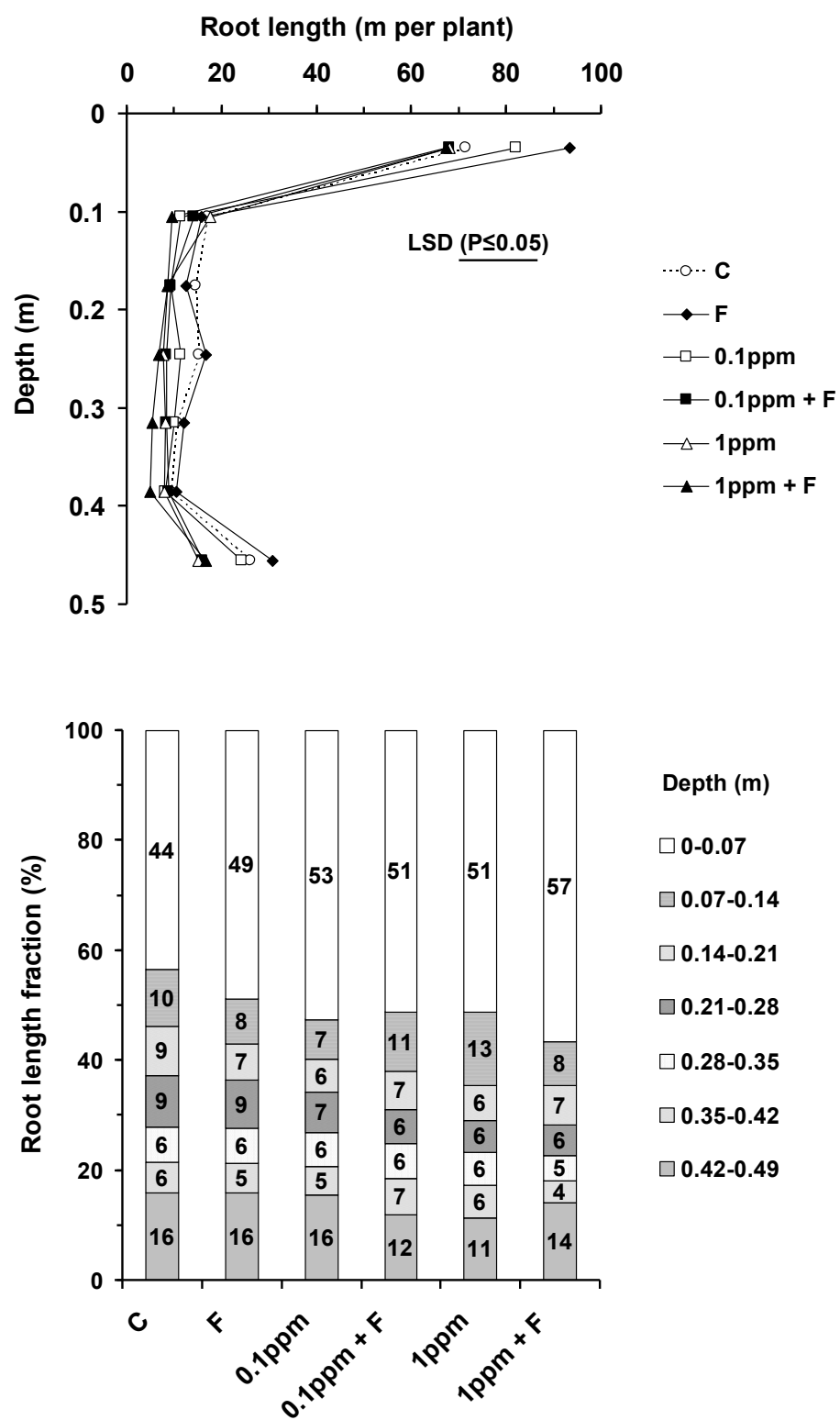


Figure 3. Profiles of root length density (RLD) (above) and distribution in various IBA treatments (below). Horizontal bar: LSD value ($P \leq 0.05$).

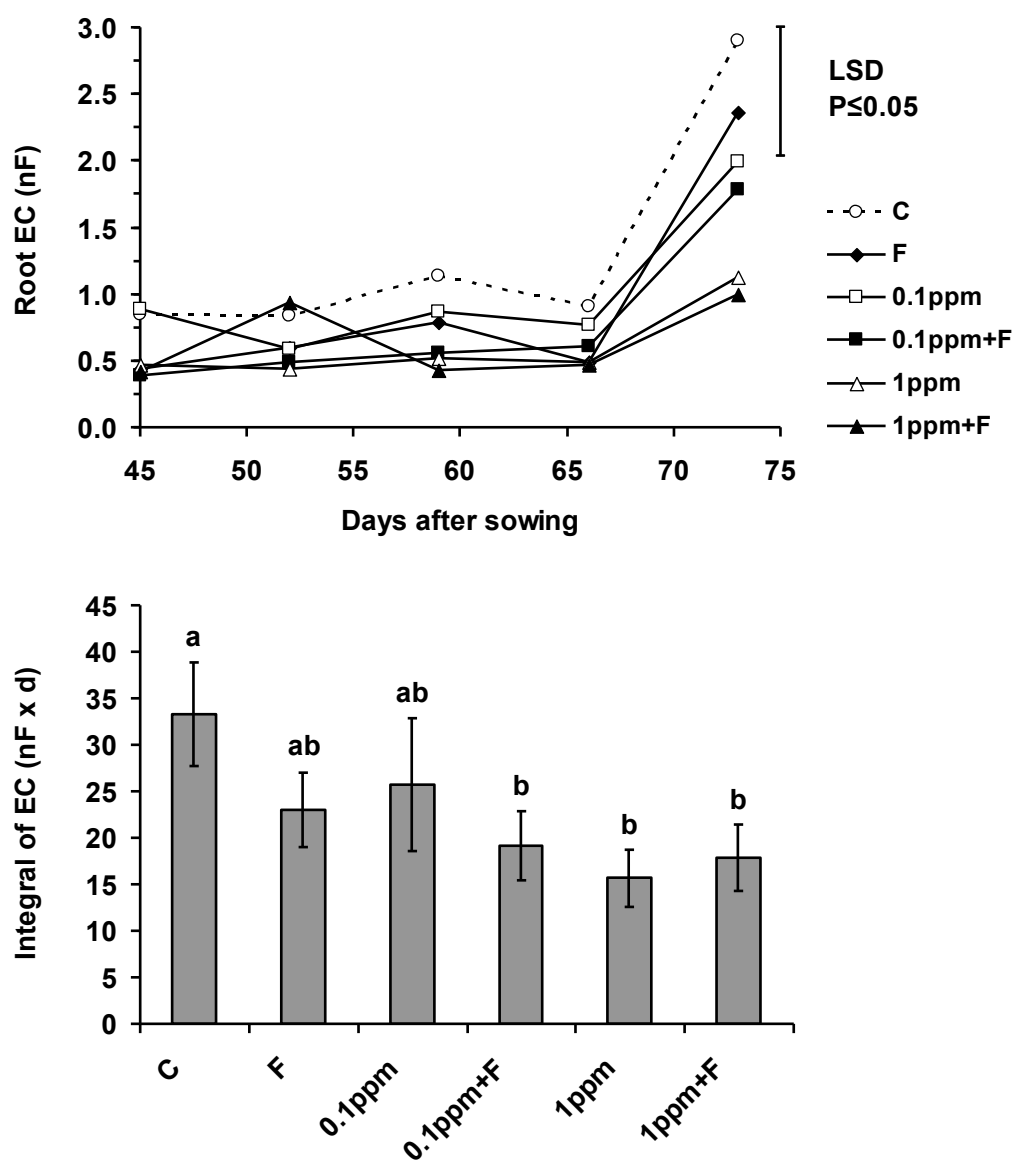


Figure 4. Dynamics of root electrical capacitance (EC) (above) and its integral over time ($\pm$ S.E., $n=5$) (below) in various IBA treatments. Vertical bar: least significant difference (LSD) for 'date' $\times$ 'treatment' interaction (0.97) ($P \leq 0.05$).

Metal uptake

Concentrations of trace elements in shoots was enhanced at 1 mg IBA kg^{-1} of soil for Co and Pb (+98% and +93%), reaching 0.55 and 4.33 mg kg^{-1} of dry biomass, respectively. In roots, Zn was again maximum for 1ppm and 1ppm+F (+43% and +67% vs. controls), the

values measured being 853 and 996 mg kg⁻¹ of dry biomass. In both shoots and roots, overall trace element concentration, i.e., summation of the 9 pollutants considered, was maximum when 1 mg IBA kg⁻¹ of soil was applied in combination with foliar treatment, the rates of increase being 29% and 36% with respect to controls, respectively (Table 2).

As a result of ‘metal concentration × biomass’, the mass of the above-limit pollutants did not differ significantly among treatments at above-ground level. The only exception was Zn, for which accumulation was lower in the 1ppm treatment (0.03 mg per plant, i.e. - 67% with respect to controls) (data not shown). When the overall metal offtakes in the whole plant were taken into account, a general trend toward reduction was measured for all IBA treatments, although it was seldom significant (0.1ppm and 1ppm) (Fig. 5).

IBA enhanced concentrations of trace elements more in shoots than in roots, with a particularly notable effect for Co and Pb – a result which implied a positive increase in the translocation factor (TF). The Co TF was particularly high at the dose of 0.1 mg IBA kg⁻¹ in combination with foliar treatment (6% vs. 3% of controls).

Table 2. Trace element concentrations ($\pm$ S.E., $n=5$) in compartments of plants cultivated in pyrite cinders with different IBA treatments. Small letters: differences among treatments within same metal (LSD test, $P \leq 0.05$). Capital letters: comparisons between shoots and roots for same element.

Treatment	As (mg kg ⁻¹)		Co (mg kg ⁻¹)		Cu (mg kg ⁻¹)		Pb (mg kg ⁻¹)		Zn (mg kg ⁻¹)		9HM (mmol kg ⁻¹)	
<i>Shoot</i>												
C	12.5±1.89	a	0.28±0.03	b	38.8±1.97	ab	2.24±0.29	b	91.4±6.91	ab	3.05±0.25	bc
F	14.8±2.15	a	0.36±0.05	ab	43.6±5.13	ab	3.39±0.49	ab	99.2±11.9	ab	3.38±0.22	abc
0.1ppm	12.1±2.92	a	0.29±0.06	b	34.1±10.5	b	2.34±0.15	b	79.1±11.9	b	2.73±0.38	c
0.1ppm+F	13.5±6.65	a	0.39±0.07	ab	41.7±6.98	ab	3.39±0.63	ab	113±6.35	ab	3.52±0.10	abc
1ppm	11.4±1.91	a	0.55±0.11	a	57.5±11.3	a	4.33±0.98	a	101±8.89	ab	3.82±0.38	ab
1ppm+F	18.9±3.71	a	0.43±0.03	ab	44.2±6.41	ab	3.17±0.52	ab	119±21.9	a	3.92±0.34	a
MEAN	13.8±1.45	B	0.38±0.03	B	43.3±3.23	B	3.13±0.25	B	100±5.28	B	3.40±0.14	B
C.V. (%)	20		26		18		25		14		13	
<i>Root</i>												
C	85.6±8.85	a	11.0±1.56	ab	506±91.4	a	307±62.5	a	596±79.7	c	22.7±3.03	b
F	68.9±7.52	a	9.09±0.83	b	563±75.0	a	379±65.5	a	676±61.6	bc	24.7±2.41	ab
0.1ppm	70.4±8.12	a	8.84±0.39	b	525±82.6	a	343±45.8	a	828±71.0	abc	27.1±1.55	ab
0.1ppm+F	88.5±24.8	a	8.87±1.70	b	548±129	a	282±40.9	a	820±88.0	abc	26.7±3.57	ab
1ppm	96.8±7.99	a	10.7±1.10	ab	537±57.7	a	405±53.1	a	853±56.4	ab	27.8±1.80	ab
1ppm+F	100±15.3	a	13.3±1.84	a	561±81.0	a	398±30.7	a	996±116	a	31.0±1.06	a
MEAN	85.65.65	A	10.40.59	A	53933.5	A	35120.5	A	79939.1	A	15.11.63	A
C.V. (%)	20		26		18		25		14		13	

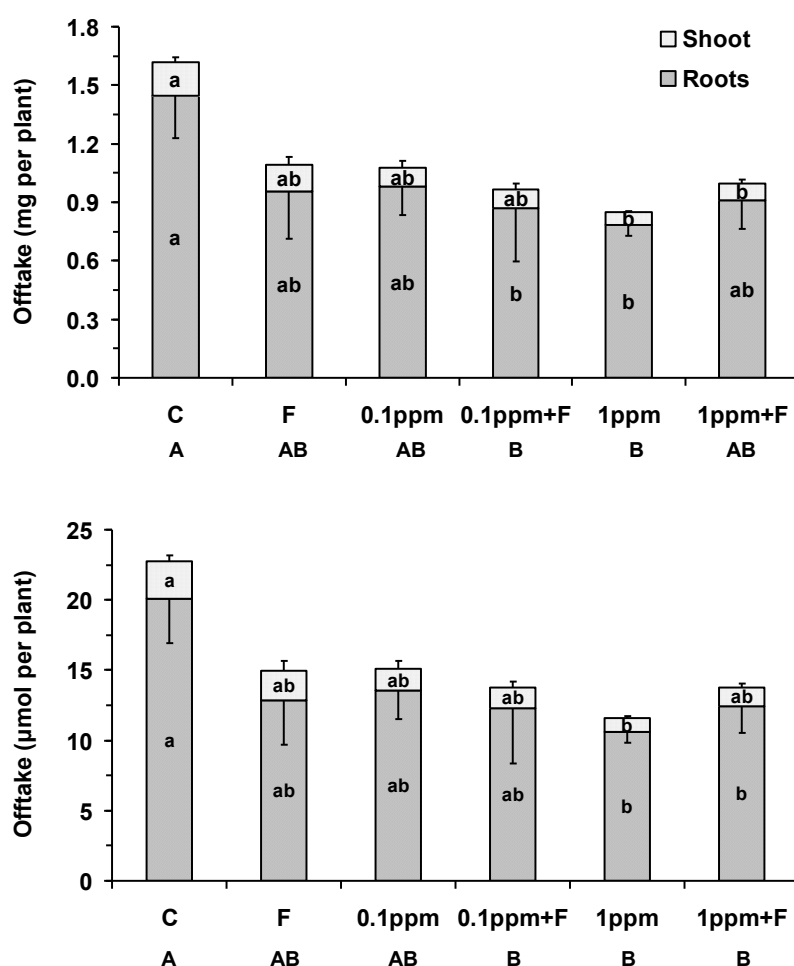


Figure 5. Overall metal uptake (As, Co, Cu, Pb, Zn, Cd, Cr, Mn, Ni) in shoot and root compartments ($\pm$ S.E., $n=5$) in various IBA treatments. Small letters: differences among treatments (LSD test, $P \leq 0.05$) within shoots and roots. Capital letters: differences among treatments for whole plant (shoot+roots) (LSD test, $P \leq 0.05$).

Among growth parameters, the overall TF of the 9 metals was positively correlated with root dry weight (DW_{roots}), which was the only variable retained by the model in stepwise regression ($R^2=29\%$):

$$TF = -0.08 + 0.61 \cdot DW_{\text{roots}}$$

In terms of mass, the shoot-to-root ratio of the 9 metals together was generally low in the experiment; compared with controls, slightly improvements were obtained with foliar application only (14% vs. 12%), and the worst with 1ppm (8%).

Water percolation and metal leaching

Volumes of water percolated during the experiment were found to be generally increased when IBA was applied to the soil – the higher the dose, the greater the effect. Percolation was even more than doubled in 1ppm+F than in control plants (Fig. 6). Only foliar application allowed water losses to be statistically similar to those of untreated controls.

Overall trace element concentrations (9 metals combined) in percolation water was lower for foliar IBA application ($2.35 \mu\text{mol L}^{-1}$) than for the 1ppm+F treatment ($3.82 \mu\text{mol L}^{-1}$), the latter reaching values 41% higher than controls (Table 3). Concerning individual metals, Pb was reduced in 0.1ppm+F, and Zn in F and 0.1ppm+F. Instead, As was greater with foliar IBA, and Cu with 0.1ppm and 1ppm+F. Co was not detectable in the water, its concentration being lower than $1 \mu\text{g L}^{-1}$. Cu, Zn and occasionally Pb did not exceed the concentration thresholds for drinking water, whereas As was always above (Table 3).

Total leaching of trace elements, calculated throughout the cycle by multiplying ‘water losses’ $\times$ ‘metal concentrations’, was increased by the application of the highest dose of IBA (1ppm), regardless of the additional foliar application. Instead, total metal moles leached of foliar application alone was equal to those of controls (Fig. 5).

The amount of percolated water was negatively correlated ($P \leq 0.001$) with both shoot biomass ($R^2=0.77$) and leaf area (integral over time) ($R^2=0.83$) and root growth (length: $R^2=0.56$, weight: $R^2=0.69$, EC integral: $R^2=0.53$), supporting the generally increased metal leaching associated with IBA treatments.

Multiple regression showed that the amount of metal lost through water percolation (ML) was significantly reduced by leaf expansion and increased by concentrations of metals in roots, as follows:

$$ML = -0.12 - 0.71 \cdot LA_{\text{int}} + 0.40 \cdot [HM]_{\text{roots}}$$

The two variables retained by the model accounted for 69% of variability, 46% by leaf area integral (LA_{int}) and 23% by root metal concentrations ($[HM]_{\text{roots}}$).

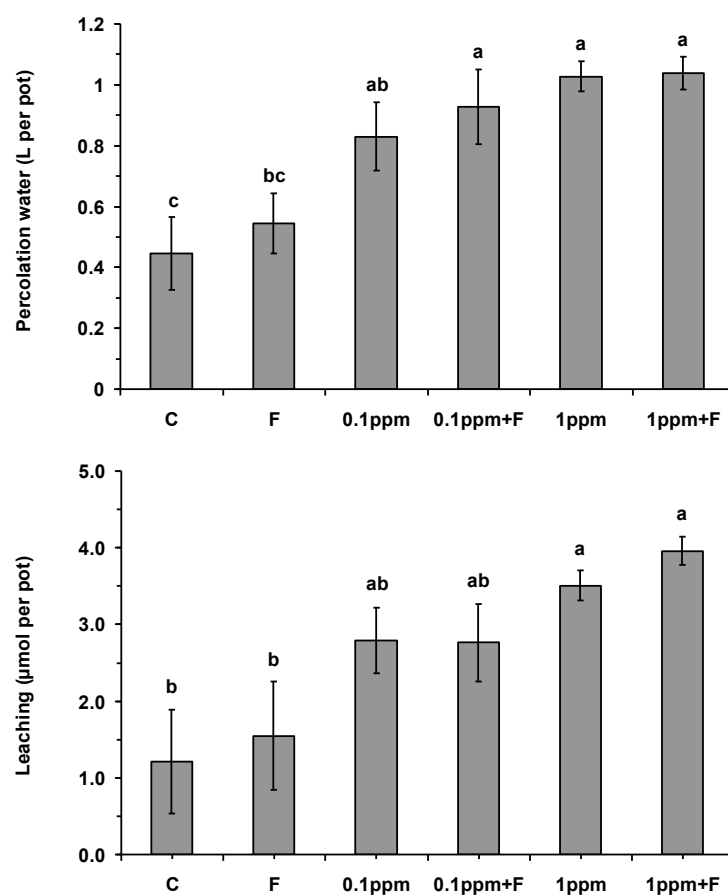


Figure 6. Volumes of water percolation (above) and overall metal leaching (As, Co, Cu, Pb, Zn, Cd, Cr, Mn, Ni) (below) ($\pm$ S.E., $n=5$) in various IBA treatments. Letters: differences among treatments (LSD test, $P \leq 0.05$).

Table 3. Concentrations ($\pm$ SE, n=5) of individual metals ($\mu\text{g L}^{-1}$) and summation of 9 elements (μM) in percolation water of IBA treatments in comparison with controls and Italian thresholds for waste water (WW, $\mu\text{g L}^{-1}$) and drinking water (DW, $\mu\text{g L}^{-1}$). Letters: differences among treatments for same metal (LSD test, $P \leq 0.05$).

Treatment	As		Cu		Pb		Zn		9 HM	
C	15.1 $\pm$ 1.63	b	79 $\pm$ 29.8	b	33.2 $\pm$ 17.8	a	56.8 $\pm$ 9.63	ab	2.71 $\pm$ 0.67	ab
F	19.2 $\pm$ 0.70	a	101 $\pm$ 44.6	ab	12.1 $\pm$ 3.45	ab	24.6 $\pm$ 4.14	c	2.35 $\pm$ 0.77	b
0.1ppm	14.0 $\pm$ 0.69	b	145 $\pm$ 12.2	a	16.7 $\pm$ 6.27	ab	36.4 $\pm$ 10.0	bc	3.33 $\pm$ 0.17	ab
0.1ppm+F	16.4 $\pm$ 1.33	ab	132 $\pm$ 17.0	ab	9.38 $\pm$ 2.31	b	24.7 $\pm$ 4.14	c	2.85 $\pm$ 0.30	ab
1ppm	15.7 $\pm$ 0.95	b	130 $\pm$ 6.57	ab	16.6 $\pm$ 5.51	ab	60.1 $\pm$ 8.05	ab	3.41 $\pm$ 0.11	ab
1ppm+F	15.4 $\pm$ 1.02	b	152 $\pm$ 10.2	a	14.9 $\pm$ 6.68	ab	64.7 $\pm$ 12.6	a	3.82 $\pm$ 0.09	a
WW threshold	50		100		100		500			
DW threshold	10		1,000		10		3,000			

Discussion

At the polluted site of Torviscosa, the pyrite wastes extended to between 0.15 and 1 m of depth, as in the past they had been covered with an 0.15-m thick layer of imported unpolluted soil. This substrate proved to be extremely inhospitable for plant establishment and growth (Marchiol et al., 2007; Vamerali et al, 2009). For this reason, and in order to improve phytoextraction, efficient soil colonisation by plant roots is needed. The use of growth-promoting substances, such as auxins, is expected to increase the amount of roots in contact with the growth medium. Indeed, the concentration activity of hormones in plant tissues are very low, and change with plant organs, with possible opposite effects (phytotoxicity) outside suitable ranges (Van Iersel, 1998). Low doses of hormones can also act like their excesses if applied repeatedly over time (Druart, 1997), causing rooting inhibition (James, 1983; Welander, 1985) and disturbances of shoot apical growth (Snir and Erez, 1980).

This study considered the suitability of improving plant growth, as a way of enhancing metal acquisition, through extended rooting, and metal accumulation through improved sink productivity. Among auxins, IBA is known to be one of the most powerful hormones in stimulating root growth, and this study is a rare attempt at its use in phytoremediation. However, within the range of dosages applied to soil, 0.5-5 mg kg⁻¹, i.e., 2.5-25 µmoles kg⁻¹ (whole plant cycle), we did not observe increases in either root biomass or in root length. Conversely, soil applications turned out to be somewhat phytotoxic at the highest rates. This was also evident from the greater fraction of root colonising the top 7-cm layer of the pot waste, which reached about 52% of total length in IBA treatments compared with 44% of controls.

The greater metal concentrations found in both roots and shoots of IBA treatments undoubtedly explain biomass losses, but a direct negative effect of the hormone on cell division and extension due to inappropriate dosage cannot be excluded. The latter hypothesis is supported by the positive enhancement of root length associated with the small dose of foliar IBA spraying. In the previous studies of Lopez et al. (2005) and

Liphadzi et al. (2006), growth inhibition was never observed with IAA in *Medicago sativa* and *Helianthus annuus*, whereas the hormone effect was positive on both root and shoot biomass but at greater dosages than we used for IBA. Compared with IAA, the activity of IBA is proved to be more persistent, as IBA is slowly oxidised in plant tissues (Epstein and Ludwig-Müller, 1993) and is less susceptible to microorganisms in soil (Hartmann et al., 1990). In *Malus*, De Klerk et al. (1997) found that the concentration of IBA in water solution at which root growth was not inhibited (10 μ M) was 3-10 times lower, with a narrow concentration range, than for IAA. These authors also pointed out that root and shoot growth was better with IAA than with IBA at the same hormone rate.

The data we acquired from this experiment make interpretation of results partially difficult, although the frequent increases in metal concentrations due to IBA in both plant compartments are very probably mediated by increases in H⁺-ATPase activity (Hager, 2003).

A generally positive morphological trait for phytoremediation resulting from IBA application was the increase in specific root length, especially for foliar treatment, indicating more efficient utilisation of plant resources below ground and greater length available for metal uptake. Indeed, *in situ* cultivation of woody species at our polluted site revealed that the finer fraction of roots is correlated with Co, Cu and Pb above-ground concentrations, but it also represented a major sink for metal accumulation. This result was also highlighted in this experiment and suggests the need to improve further metal translocation, for instance by combining auxins with chelators, as done by Liphazi et al. (2006) with IAA.

The IBA soil doses we managed in this experiment should be used with caution, as they were responsible for significant metal leaching – a result we attribute to marked reduction of leaf expansion and transpiration potential. Interesting was the small leachate of IBA foliar spraying, which indicates the need of further studies of the above-ground applications of this hormone and other auxins for profitable and safe use in phytoremediation. In this regard, auxins may also enhance transpiration through stomatal opening, although identification of treatment concentration is very critical, as shown by Russell et al. (2006). In *Pisum sativum*, these authors found 68% of stomata open in

comparison with only 15% of untreated leaves under 1 μM IAA, but the percentage fell to 20% when the IAA dose was 0.1, 10, or 100 μM .

Conclusions

At the IBA rates tested in this experiment, auxin did not produce profitable increments in metal removal, by either harvestable shoot or root biomass. Instead, decreased efficiency of phytoremediation at high IBA doses is to be expected. Most of these results are due to worsening in yield productivity and root phytotoxicity, occurrences that may give rise to the risk of metal contamination of groundwater.

Increased concentrations in tissues of some metals, such as Co and Pb in shoots and Zn in roots, suggests that IBA probably acts below-ground more on H^+ -ATPase than on cell division and growth. The only exception of foliar spraying (minimum IBA dose), indicates that dosages were very probably excessively high, thus exerting a negative effect on growth. The promising effects of IBA on rooting through foliar application should be better investigated, in order to identify more profitable dosages to be given to the canopy and not through soil irrigation, in comparison with other plant-promoting substances.

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Chapter VI. Root phytotoxicity and metal leaching in EDDS-assisted phytoextraction with *Brassicaceae* species⁴

Abstract

This study examines the influence on root growth and metal leaching of dose and application time of EDDS, a more recent and less persistent chelator. In a pot experiment, Ethiopian mustard (*Brassica carinata* A. Braun) and fodder radish (*Raphanus sativus* L. var. *oleiformis*) were cultivated in pyrite wastes severely contaminated by As, Co, Cu, Pb and Zn.

Plants were grown for 75 days, and four EDDS treatments were compared with untreated controls. Doses were 2.5 and 5 mmol EDDS kg⁻¹ soil applied one week before harvest, and 1 mmol EDDS kg⁻¹ soil repeated five times at 5- and 10-day intervals. Fodder radish treated with 1 mmol at the 5-day interval was also added with 1 mg IBA (indole-3-butyric acid) per kg of soil every 10 days.

Shoot biomass, leaf area and root length were generally reduced in EDDS-treated plants of both species, particularly in repeated treatments and radish, regardless of the application of IBA. The weight of roots was more affected by EDDS than length and electrical capacitance. Overall metal leaching was explained by a multivariate relationship ($R^2=0.52$), which included the integral over time of both leaf area and root electrical capacitance as independent variables, accounting for 43% and 9% of variability, respectively. We conclude that roots play a secondary role in preventing metal leaching, because of the prevailing effect of leaf transpiration in reducing volumes of percolation

⁴ From: Bandiera M., Mosca G., Vamerali T (2009) Root phytotoxicity and metal leaching in EDDS-assisted phytoextraction with *Brassicaceae* species. Plant Biosyst., accepted with minor revisions.

water. The best metal phytoextraction was achieved with EDDS applications at harvest, a method that also did not increase either water percolation or metal leaching, especially at the low dose of 2.5 mmol kg⁻¹ of soil, unlike earlier low-dosage treatments.

Abbreviations: EC: electrical capacitance; EDDS: ethylene-diamine-disuccinic acid; EDTA: ethylene-diamine-tetraacetic acid; HM: heavy metals; IBA: indolebutyric acid; ICP-OES: inductively coupled plasma – optical emission spectroscopy; LA: leaf area; ML: metal leaching.

Introduction

Phytoextraction exploits the ability of plants to take up pollutants, such as heavy metals, from the soil for land reclamation purposes (Raskin et al., 1994). Although many attempts have been made to improve the efficiency of this technology, scarce metal availability in the soil for uptake and modest translocation from roots to harvestable biomass still remain important constraints.

The application of various kind of chelators to increase heavy metal mobilisation from the soil solid phase to the bulk pore water has been proposed in assisted phytoextraction with biomass species (Blaylock et al., 1997; Grčman et al., 2001). Due to the slow uptake of metals by plants, the use of chelators has always been considered environmentally dangerous, causing possible metal leaching into groundwater. For this reason, the choice of chelator and dose is an important task. Ethylene-diamine-tetraacetic acid (EDTA) has been intensively tested in phytoextraction, but its persistence in the environment and toxic effects on soil markedly limit its applications (Grčman et al., 2001). Metal losses may greatly exceed the amount taken up by plants after EDTA application,

suggesting that its safe use is restricted to hydrologically controlled areas (Wenzel et al., 2003).

Among chelators, ethylene-diamine-disuccinic acid (EDDS), a structural isomer of EDTA, has recently received increased attention. EDDS is an aminopolycarboxylic acid naturally produced by some microorganisms (Goodfellow et al., 1997), with low persistence in soil. Schowanek et al. (1997) found that the [S,S]-EDDS stereoisomer is rapidly and completely mineralised, with a half-life ranging from 2.5 to 4.6 days, depending on type of medium and associated metal (Vandevivere et al., 2001). In soil washing, at neutral soil pH EDTA extracts higher percentage of Cu, Zn and Pb than EDDS when the chelator-to-metal concentration ratio is relatively high (>10). Conversely, at equimolar concentrations, metal extraction due to EDDS is greater than that of EDTA for Cu and similar for Zn and Pb, due to the lower affinity of EDDS for Ca (Tandy et al., 2004). The stability constant of metal-EDDS complexes at pH 7 follows the order $\text{Fe} > \text{Cu} > \text{Zn} > \text{Pb}$, and this may affect metal extraction in cases of pluricontamination. In particular, the affinity for Cu is very similar between EDDS and EDTA (log K: 18.4 vs. 18.8) (Tandy et al., 2004).

In some species, water and solutes can enter the xylem through unsuberised endodermal cells, called passage cells (Clarkson, 1996). Metal-chelator complexes can enter the xylem by passing along an apoplastic pathway and through breaks in the Casparian strip (Tanton and Crowdy, 1971). However, at high rates (5 mmol kg^{-1} soil) EDDS may become phytotoxic, and it is likely that the physiological barriers in roots may be destroyed (Luo et al., 2005).

The few studies available on the use of EDDS show contradictory effects on shoot growth, whereas very little information is available on its influence on roots and metal leaching. The shoot biomass of *Brassica rapa* L., *Cannabis sativa* L., *Helianthus annuus* L., and *Zea mays* L. was not reduced when plants received $3 \text{ mmol EDDS kg}^{-1}$ soil four weeks before harvesting (Meers et al., 2005). Instead, marked phytotoxic effects were observed in *Zea mays* L. and *Phaseolus vulgaris* L. two weeks after the application of 5 mmol kg^{-1} EDDS (Luo et al., 2005). Efficient application of the same EDDS dose was experimented in *Brassica carinata* A. Braun in a pluricontaminated soil, allowing from 2- (Zn) to 4-fold (Cu, Pb) increased concentrations in shoots compared with controls

(Quartacci et al., 2007). These authors did not find shoot phytotoxicity by applying EDDS one week before harvest in pots, but root length and the shoot-to-root ratio were reduced in hydroponics.

This experiment aims at revealing the possible root phytotoxicity of EDDS at various doses and times of application, and at establishing the role of roots in preventing metal leaching in assisted phytoextraction with *Brassicaceae* species. Data were collected from a pot experiment, growing Ethiopian mustard and fodder radish in pluricontaminated metal-polluted pyrite wastes.

Material and methods

Pot experiment set-up

Fodder radish (*Raphanus sativus* L. var. *oleiformis*) and Ethiopian mustard (*Brassica carinata* A. Braun) were cultivated in a greenhouse for 75 days. Cylindrical opaque PVC pots, with an inner diameter of 57 mm and height of 520 mm, were caulked with pipe insulation sheaths. Pots were filled to a depth of 490 mm with a mixture of metal-polluted pyrite cinders (Table 1) and sand (1:1 w/w) and their bottom was connected with black tubes to opaque PVC bottles (1 L) in order to collect leachate. One plant per pot was sown at the end of May and regularly (about twice a week) watered with 1:2 diluted Hoagland solution. The cinders had been dumped in the past as the by-product of pyrite ore roasting for sulphur extraction at Torviscosa (Udine, north-east Italy), within an area which is part of the site of national interest for reclamation called ‘Lagoon of Grado and Marano and adjacent rivers’. The cinders were severely contaminated by As (886 mg kg⁻¹), and Co, Cu, Pb and Zn (100, 1,735, 493 and 2,404 mg kg⁻¹, respectively), largely exceeding Italian legal

limits for agricultural uses (Table 1). Organic matter content was very low, pH was 7.3, and electrical conductivity 0.3 S m^{-1} .

Compared with untreated controls (C), four EDDS treatments were examined: a low dose of 2.5 mmol kg^{-1} soil (called 2.5) and a high dose of 5 mmol kg^{-1} soil (called 5) applied one week before harvest, and a dose of 1 mmol kg^{-1} soil repeated five times at 5- (1x5-5d) and 10-day (1x5-10d) intervals, starting from 48 and 28 days after sowing (DAS), respectively. In an attempt to improve root growth, fodder radish treated with 1 mmol EDDS at the 5-day interval was also added with 1 mg IBA (indole-3-butyric acid) kg^{-1} soil at 10-day intervals (EDDS+IBA).

A 5 M stock solution of EDDS was prepared and stored in the dark at 4°C . As each pot weighed 2 kg , $1 \text{ mmol EDDS kg}^{-1}$ of soil was applied by taking 0.4 mL of stock solution diluted in 100 mL of water, an amount that did not cause percolation. Doses 2.5 and 5 mmol were given by proportionally increasing the amount of initial solution. Untreated plants received equivalent amounts of distilled water. Each pot received 3.8 L of water (Hoagland, EDDS treatment, water) throughout the cycle.

Table 1. Concentrations for above- and some below-limit metals in pyrite wastes, in comparison with Italian legal limits (Italian legislative decree 152/2006, for public, private and residential areas).

Metal	Total content (mg kg^{-1}) (A)	Legal limit (mg kg^{-1}) (B)	(A)/(B)	DTPA- extractable (mg kg^{-1})
As	886	20	44/1	-
Zn	2404	150	16/1	44.6
Cu	1735	120	14/1	142
Co	100	20	5/1	-
Pb	493	100	5/1	69.8
Cd	0.46	2	1/4	0.23
Ni	3.1	120	1/38	0.16
Cr (total)	2.5	150	1/61	-
Mn	108	-	-	-

Shoot measurements

During the growth period, leaf expansion was monitored at weekly intervals, starting on 11 July (47 DAS, days after sowing). Values of leaf area were obtained from non-destructive measurements of leaf diameters during the cycle, and by means of a LI-3100 leaf area meter (Li-Cor Inc., Lincoln, Nebraska, USA) at harvest. Final shoot biomass was measured after oven-drying at 105 °C for 24 hours and metal contents revealed through ICP-OES (Inductively Coupled Plasma - Optical Emission Spectroscopy).

Root investigation

Root EC was recorded during the cycle at the same time interval as leaf measurements by a hand-held digital ELC-131D electrical capacitance meter (Escort Instruments Corporation, Taipei, Taiwan) (Preston et al., 2004). After preliminary calibration, the following settings were adopted:

a) positively-charged electrode (stainless steel rod, 2 mm diameter) 0.55 m long, 0.3 m inserted into the potting soil (vertical), and cable connection 60 mm above-ground level, earthed 20 mm away from the stem base;

b) negatively-charged electrode: fine stainless steel needle, orthogonally inserted into the stem at a height of 10 mm (1 mm penetration into the stem).

Capacitance values (pF) were recorded at 1 kHz frequency, 24 h after watering (200 mL Hoagland solution per pot), to reach approximate field capacity and to allow roots to come into contact with the soil solution.

At harvest, roots were separated from soil through flotation by means of a hydraulic centrifugation device and collected in a 500-µm mesh sieve, as described by Oliveira et al. (2000). Separation of soil particles was facilitated by the addition of oxalic acid solution (2% w/v). Roots were stored at 4 °C in an ethanol solution (10% v/v) until analysis. One-bit 400-DPI TIFF-format images of roots were acquired by digital scanning on a flatbed scanner (EPSON Expression 10000XL, Canada). Root length was measured by KS 300 image analysis software (Carl Zeiss Vision GmbH, München, Germany) following the procedure of Vamerali et al. (2003). Discrimination of roots against extraneous objects was

performed according to an Elongation Index value (i.e., perimeter²/area) greater than 60 and a minimum object area greater than 25 pixels. Root length (*FbL*) was derived from perimeter (P) and area (A) of the digital objects, as follows:

$$FbL = \frac{P + \sqrt{P^2 - 16A}}{4}$$

Subsequently, root dry mass and its metal contents were determined as for shoots.

Percolation water

Volumes of percolation water, collected throughout the experiment, were measured at harvest, and ICP-OES analysis allowed the calculation of metal leaching.

In both plant tissues and water, besides the five above-limit metals, concentrations of Cd, Cr, Mn, and Ni were also revealed.

Statistical analysis

Statistical analysis was carried out by Statgraphics software (Manugistic Inc., Rockville, MD, USA). ANOVA and the LSD test ($P \leq 0.05$) were used to evaluate differences among means for growth parameters and metal concentrations.

Stepwise regression with both forward and backward variable selection was applied to overall metal leaching in relation to seven independent variables, i.e., shoot dry weight, leaf area (integral over time), root dry weight, root length, root EC (integral over time) and metal concentrations in shoots and in roots. Before analysis, the data of each variable were standardised by subtracting the mean and dividing by standard deviation, so that the regression coefficients of variables in the model were comparable.

Results

Growth parameters

The harvestable biomass of plants treated with EDDS was generally reduced compared with untreated controls, especially for repeated applications (Fig. 1). Phytotoxicity was less evident in Ethiopian mustard than in fodder radish, showing a reduction of 42% and 54% (average of all EDDS treatments, EDDS+IBA excluded), respectively.

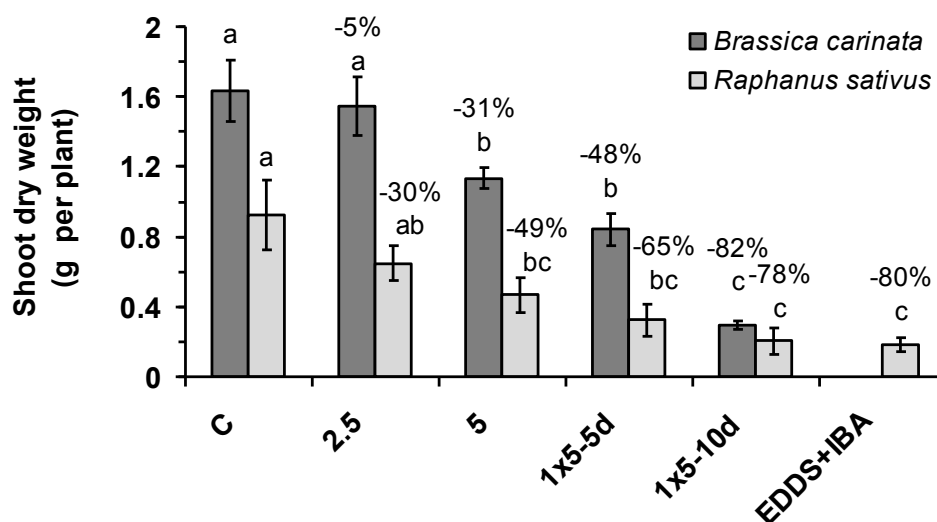


Figure 1. Shoot biomass at harvest ($\pm$ SE) in different treatments and percentage variation compared with controls. Letters: differences among treatments within same species (LSD test, $P \leq 0.05$).

EDDS was also phytotoxic below ground, particularly in radish, as observed in shoots. In mustard, root length was reduced only when EDDS was given very early (1x10-10d treatment, -59%), whereas in radish decreases were measured for repeated treatments (-49% and -60% for 5- and 10-day application intervals, respectively) and at the maximum

dose (5 mmol, -42%) (Fig. 2). Similar results were found when root electrical capacitance was considered. The dynamics of EC followed the same trend in the two *Brassicaceae*, with markedly reduced values for 1x5-10d treatment (Fig. 3). In mustard treated with EDDS at 5-day intervals, root length was found somewhat increased, although this was not evident from the EC values. Despite this, there was a good correlation between root EC (integral over time) and both length (R^2 : mustard 0.63, radish: 0.58, $P \leq 0.01$) and weight (R^2 : mustard 0.74, radish: 0.36, $P \leq 0.01$) of roots.

The additional application of IBA in radish did not produce any improvements in growth at either above- or below-ground compartments. In general, biomass partitioning within plants was not influenced by EDDS, and the root-to-shoot ratio decreased from a normal value of about 1 to 0.55 in radish only when plants received EDDS close to harvest.

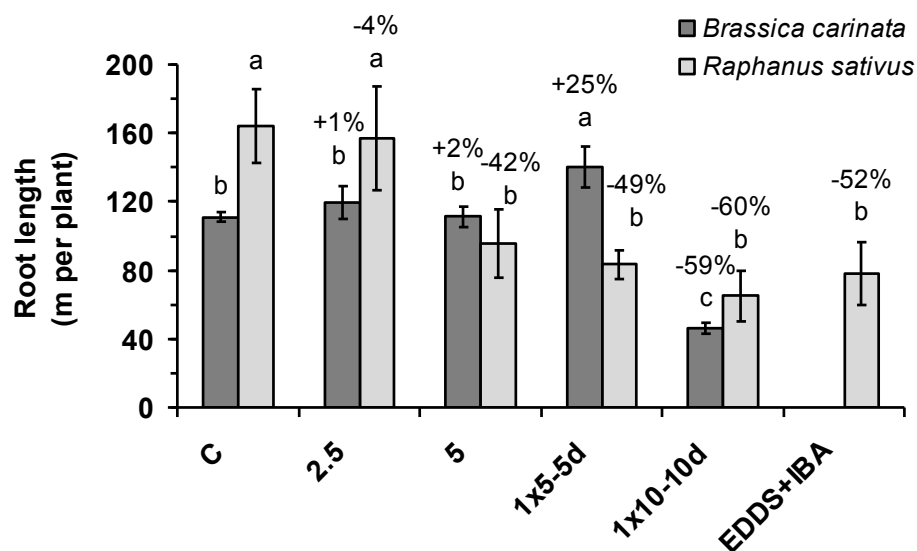


Figure 2. Total root length at harvest ($\pm$ SE) in treatments of two species and percentage variation compared with controls. Letters: differences among treatments within same species (LSD test, $P \leq 0.05$).

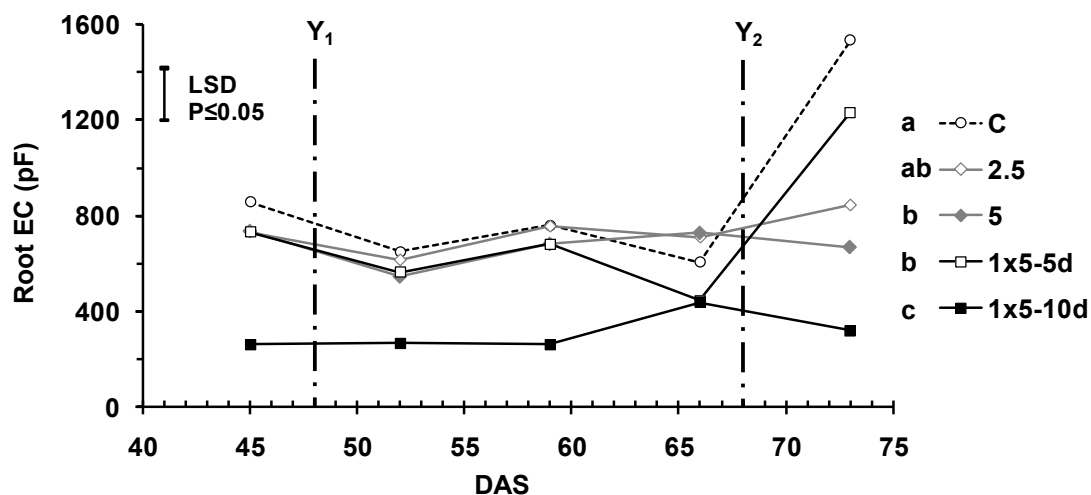


Figure 3. Dynamics over time (days after sowing) of root electrical capacitance (EC) in *Brassica carinata*. Letters: differences for 'treatment' main effect (LSD, $P \leq 0.05$). Vertical bars: LSD for 'treatment $\times$ time' interaction (217); Y_1 = time of first EDDS application (1x5-5d); Y_2 = time of EDDS application for 2.5 and 5 treatments.

Metal uptake

In shoots, overall metal concentrations, i.e., summation of the nine considered pollutants, were always increased by EDDS, particularly in radish (4.9 vs. 3.5 mmol kg⁻¹ of mustard, average of EDDS treatments) and in repeated treatments (Fig. 4). Increases regarded Cu and Co in both species, plus Zn in mustard and Pb in radish. For Cu and Co, the maximum increment was detected in the 1x5-5d treatment, with about 3-fold greater concentrations, reaching 160 and 1.27 mg kg⁻¹ respectively.

As a result of 'harvestable biomass × metal concentrations', total removal of various metals by shoots was higher in mustard compared with radish (3.3 vs. 1.9 μmol per plant). Mustard treated with 2.5 mmol of chelator only achieved higher removal values than controls (Fig. 4). In radish, EDDS did not improve phytoextraction, and in both species worse results were revealed for the earliest EDDS application (1x5-10d).

In controls and in EDDS treatments, metal concentrations in root tissues were several times higher than in shoots, but translocation to the above-ground biomass was generally improved by the chelator, particularly for Cu, Co and Zn (data not shown).

Root growth was found to play an important role in metal uptake, as highlighted by the positive correlations ($P \leq 0.01$, two species) between both root length and EC and total amount of metals stored in the plant (shoot+roots) (all data). Considering the two species together, the coefficients of determination were 0.46 and 0.29, for root length and root EC, respectively.

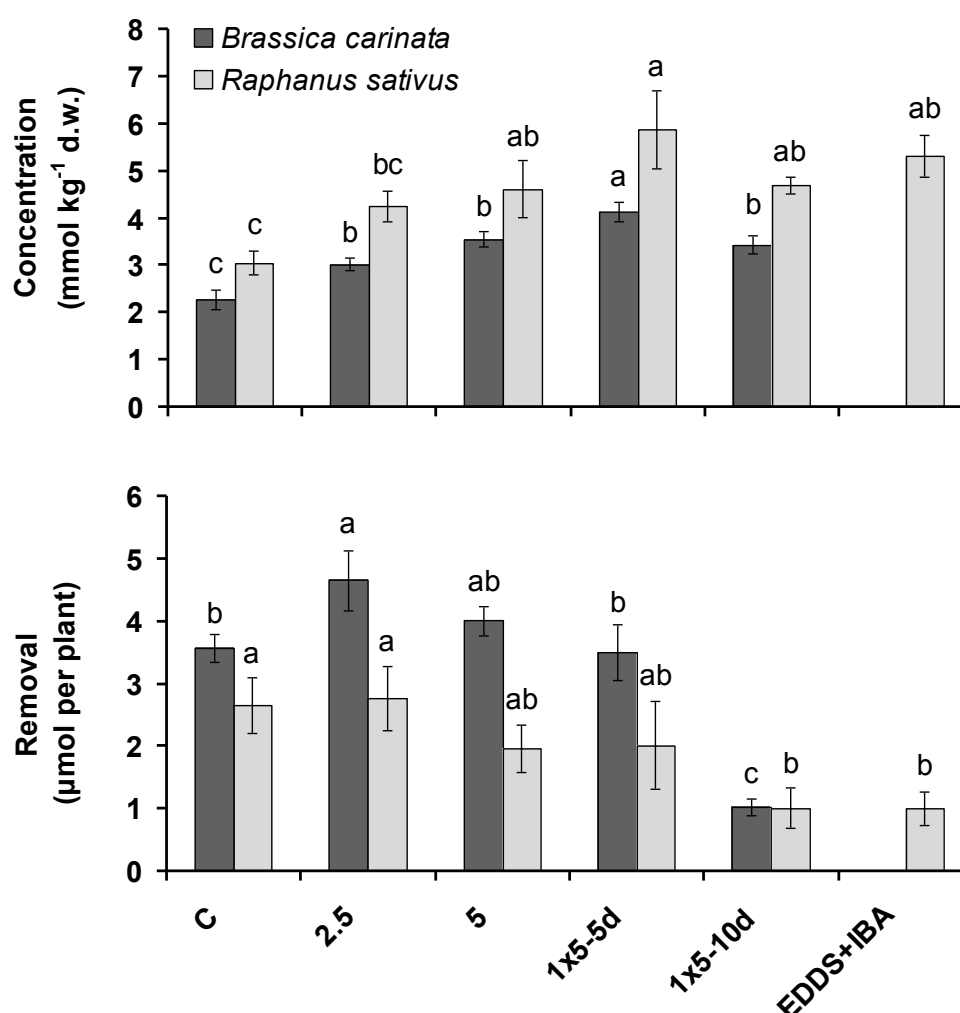


Figure 4. Total concentration (above) and removal (below) in shoots of various metals (summation of As, Co, Cu, Pb, Zn, Cd, Cr, Mn, Ni) ($\pm$ SE) in treatments of two species. Letters: differences among treatments within same species (LSD test, $P \leq 0.05$).

Water percolation and metal leaching

Volumes of percolation water collected throughout the cycle generally increased as a consequence of EDDS amendment in both species, although variations were significant for repeated treatments only (1x5-5d, 1x5-10d).

The amount of water lost by wastes was negatively correlated ($P \leq 0.01$) with shoot weight ($R^2 = 0.63$ and 0.78 for *B. carinata* and *R. sativus*, respectively) and the integral of leaf expansion ($R^2 = 0.64$ and 0.68). Poorer root EC (integral) and length were also

important causes of water percolation (EC: $R^2=0.89$ and 0.65 ; root length: $R^2=0.72$ and 0.41) and metal leaching (EC: $R^2=0.74$ and 0.28 ; root length: $R^2=0.67$ and 0.14 , for the two species respectively).

Compared with controls, repeated EDDS applications caused significant loss of metals from the cinders into percolated water (Fig. 5). Most of the leachate was accounted for by Zn and Cu (about 40 and 45%, respectively) in the case of EDDS application at harvest, and by Cu (up to 99%) in repeated treatments.

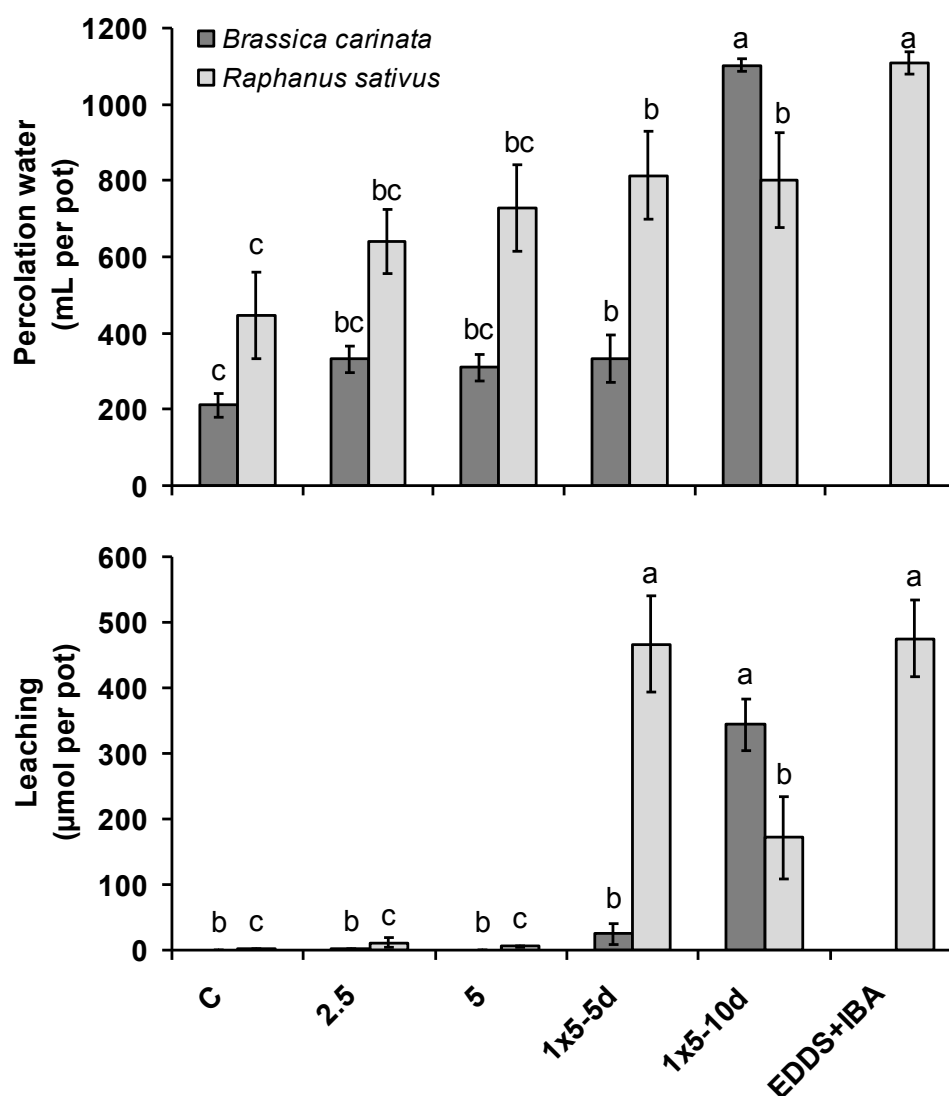


Figure 5. Volumes of percolation water (above) and leachate (9 metals) (below) ($\pm$ SE) in treatments of two species. Letters: differences among treatments within same species (LSD test, $P\leq 0.05$).

In controls, water percolation fulfilled the more restricted thresholds for drinking water for almost all metals, except As and Pb (Table 2). EDDS worsened the quality of water, especially for Cu under repeated applications. Arsenic, Pb and occasionally Ni were common contaminants of water, regardless of the use of EDDS.

A multiple regression showed that the amount of metal loss through water percolation (ML) was significantly influenced by root electrical capacitance and leaf area only. The two variables retained by the model accounted for 52% of variability, 9% by EC integral (EC_{int}) and 43% by leaf area integral (LA_{int}), as follows:

$$ML = -0.06 - 0.53 \cdot LA_{int} - 0.33 \cdot EC_{int}$$

Table 2. Single ($\mu\text{g L}^{-1}$) and total ($\Sigma[\text{HM}]$, $\mu\text{mol L}^{-1}$) metal concentrations ($\pm\text{SE}$) in percolation water of treatments in two species in comparison with Italian thresholds for waste water (WW) and drinking water (DW). Letters: differences among treatments within same species (LSD test, $P \leq 0.05$).

Missing values: concentrations below detection limit (Cd, Co, Cr: $1 \mu\text{g L}^{-1}$; Pb: $2 \mu\text{g L}^{-1}$).

Metal	WW	DW	Species	C	2.5	5	1x5-5d	1x5-10d	EDDS+IBA
As	50	10	<i>B. carinata</i>	13.2ab	17.2a	11.2b	17.2a	12.2ab	-
			<i>R. sativus</i>	15.1ab	15.6a	11.8ab	10.6b	13.0ab	12.6ab
Zn	500	3000	<i>B. carinata</i>	40.8b	167a	44.4b	45.8b	45.2b	-
			<i>R. sativus</i>	56.8bc	122ab	128a	61.8abc	24.8c	93.6abc
Cu	100	1000	<i>B. carinata</i>	12.6b	33.0b	13.8b	4469b	19650a	-
			<i>R. sativus</i>	79.1d	876d	328d	38025a	11514c	26760b
Co	-	50	<i>B. carinata</i>	-	-	-	18.0b	68.6a	-
			<i>R. sativus</i>	-	3.00c	2.00c	76.0a	70.8ab	54.2b
Pb	100	10	<i>B. carinata</i>	-	9.00	28.0	19.0	-	-
			<i>R. sativus</i>	33.2ab	40.0a	41.2a	-	28.0a	97.0a
Cd	-	5	<i>B. carinata</i>	-	-	-	-	-	-
			<i>R. sativus</i>	-	-	-	-	-	-
Ni	200	20	<i>B. carinata</i>	4.67bc	3.00c	3.00c	10.5b	20.2a	-
			<i>R. sativus</i>	8.22c	15.0b	9.80c	26.8a	21.0ab	21.8ab
Cr (total)	1000	50	<i>B. carinata</i>	-	-	-	-	-	-
			<i>R. sativus</i>	-	-	-	-	-	-
Mn	200	50	<i>B. carinata</i>	6.00ab	33.2a	4.67a	5.80a	4.00a	-
			<i>R. sativus</i>	8.86b	8.00b	8.20b	55.8a	3.60b	69.3ab
$\Sigma[\text{HM}]$			<i>B. carinata</i>	1.13b	3.97b	1.16b	71.7b	312a	-
			<i>R. sativus</i>	2.71d	16.5d	7.81d	602a	183c	425b

Discussion

EDDS is a relatively new chelator with fast degradability in soil, the introduction of which may potentially replace the more toxic and persistent EDTA in metal phytoextraction. Although some reports claim that its phytotoxicity is very small (e.g., Meers et al., 2005), we observed a general reduction of growth in two *Brassicaceae* species at both above- and below-ground levels. At high rates, EDDS exerts its phytotoxicity by damaging the membranes of root cells (Luo et al., 2005), but this would not explain our worse growth results at repeated low doses. It is probable that the top application of EDDS causes a temporary high concentration in the shallow layers of our pots, leading to intense desorption of metals in sensitive young plants. EDDS positively affects uptake and translocation of metals, but in our experiment this only rarely implied better metal removals. Impaired growth should be considered as a direct consequence of greater metal accumulation in plants, and this negatively influences metal removals.

Unexpectedly, the application of auxins to the wastes was ineffective on plant growth, but similar results were highlighted by Liphadzi et al. (2006), who found a positive effect of IAA on root growth only when EDTA was not given. Although the dose of IBA applied to our plants may have been excessively high, able to cause a reduction in growth, the results of the above authors indicate a possible interaction between auxins and chelators.

The biomass of shoots was reduced by EDDS to about the same extent as of roots. Instead, root length and electrical capacitance were less sensitive, suggesting that root morphological changes may be necessary for plants as a survival strategy, as occurs for other abiotic soil stresses. Root biomass is more affected than shoot only in some cases in the literature, as found for instance in *Vetiveria zizanioides* (L.) Nash. treated with 0.8 mmol kg⁻¹ EDTA (Weihong et al., 2009). In our experiment, root length was an important indicator of root activity, being well correlated with metal uptake (overall accumulation, shoot+roots). This result is due to the essential role in uptake and storage of metals exerted by roots. Electrical capacitance was also a significant index of root activity and mainly

affected the amount of metal leaching. The multivariate function we found for explaining metal losses through percolated water included the integral over time of both leaf area and electrical capacitance. The model allowed us to quantify the role of these two variables and establish the major role of leaf expansion. The important role of the canopy in reducing the downward movement of water and metal leaching through transpiration is widely recognised in both trees and herbaceous species (Schmidt, 2003; Menon et al., 2005). Until now no studies have quantified its importance compared with other variables, and there is need to gain a good leaf expansion before the application of chelators.

Irrespective of the application of chelators, pyrite cinders themselves are hazardous for metal leaching, since As and Pb are common leached metals, as found for this kind of waste by Ross (1994). In the polluted site from which we collected the wastes, metal leaching was prevented by a thick layer of clay at depth (Vamerali et al., 2009), but verification should be made on the safety of assisted phytoextraction. In pots, we detected severe water contamination by Cu and Zn, depending on the application time of the chelator. This is a result of the high affinity of EDDS, mainly for Cu and secondly for Zn, which were also the two most important pollutants in the plants.

Phytotoxic effects and metal leaching are affected by species choice. In the two *Brassicaceae* considered here, shoot dry weight was reduced at the same rate by EDDS, whereas mustard was less sensitive at root level, for both biomass and length. Harvest took place during stem elongation, but growth was not comparable between species, due to differences in precocity. We expect that the faster initial growth of mustard reduces water percolation in Bioavailable Contaminant Stripping (BCS) (our controls) as well as in assisted phytoextraction. Radish has previously been cultivated in pyrite wastes with good phytoextraction results (Bandiera et al., 2009), but several authors have shown its rapid senescence under EDTA and EDDS amendment (e.g., Kos et al., 2003) and this would support our results on the reduction of the root/shoot ratio under abundant EDDS amendment at harvest.

These results show that a single, moderate dose of EDDS (2.5 mmol kg⁻¹) applied before harvest improves the phytoavailability of metals without causing the risk of metal leaching into groundwater, with some attention to interspecific variability and tolerance to pollution of plant species. At the chelator doses used here (5 mmol EDDS kg⁻¹ of soil as

maximum), the EDDS-to-metal concentration ratio was generally below 1, with the exception of Co and Pb. Since the two doses tested at harvest did not significantly modify metal losses, we may expect that metal leaching and its composition are not influenced by the EDDS-to-metal concentration ratio, although within reasonable limits. Further investigation is however needed to forecast possible leaching after plant harvest.

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Chapter VII. Phytoremediation of canal sediment by inorganic amendment: effect on metal mobility

Abstract

This study describes the phytoremediation of a contaminated canal sediment in north-western England (United Kingdom). The sediment was contaminated by heavy metals and organic pollutants from various industries and shipping. After the canal was closed to navigation in 1978, the water level progressively fell from more than 3 m to its current depth of about 1.5 m, and the exposed sediment has been colonised by spontaneous vegetation.

In a previous three year-trial (started in 2002), hybrids and clones of willow (*Salix*), poplar (*Populus*) and alder (*Alnus*) were planted in the sediments of the dredged canal, for phytoextraction and stabilisation purposes. After a period of abandon, in the present study (carried out in 2008), sediments and plant tissues were analysed to evaluate further the clean-up effectiveness of planted species in comparison with the original spontaneous vegetation. A good degree of establishment was found for all species, but with maximum metal concentrations in *Salix atrocinerea* only, a species that was also growing spontaneously. Leaf tissues usually showed higher accumulations of trace elements than woody ones.

The possibility of immobilising trace elements (Cd, Cu, Pb, Zn) in the sediment was also explored by means of various inorganic amendments. In the laboratory, the sediment was mixed with cement at 1, 20, and 50% w/w, lime at 1% w/w, and lime+Fe₂(SO₄)₃ both at 0.5% w/w. In order to simulate natural conditions, in which the canal sediment were exposed to alternating wetting and drying cycles by varying the water level, a contiguous wet-and-dry extraction (4 cycles) was performed, for comparisons with untreated controls.

Results showed that Zn was efficiently immobilised by cement, regardless of the dose used, unlike lime+Fe₂(SO₄)₃ which resulted in early high mobilisation during the first cycle. Lime+Fe₂(SO₄)₃ also solubilised substantial amounts of Cd, and high cement rates of Cu. Lime alone and the smallest rate of cement were effective towards almost all metals. Pb mobility could not be controlled by any of the tested amendments.

It is concluded that reduced risks of metal leaching in phytomanagement of canal sediments should effectively consider amendments with cement or lime at small doses, with due care and attention in the case of lime, which is known to mobilise arsenic.

Abbreviations: BAC: biological absorption coefficient; LOI: loss on ignition; TOC: total organic carbon.

Introduction

Trace elements contaminating water bodies are most frequently immobilised in bottom sediments, as natural sedimentation processes tend to retain most chemical pollutants entering waterways (Bramley and Rimmer, 1988). However, when environmental conditions change, metals may easily be released (Du Laing et al., 2009) and their fate should be carefully monitored in order to prevent wider spreading. For instance, Tack et al. (1996) showed that the solubility of most metals, such as Cd, Cu, Pb and Zn, strongly increases in oxidising environments, as a function of variations in pH. Instead, As is released into solution in anoxic conditions, and is removed from solution through adsorption and precipitation processes when sediments become oxidised (Hartley et al., 2009).

The environmental policy of the European Union encourages sediment valorisation but, currently, dredged sediments are often too contaminated to be usable.

Phytoremediation may have a potential for contaminated sediment treatment, especially for trace elements, in view of its low cost and enhancement of the aesthetic quality of the landscape (Schwitzguébel et al., 2002).

A vegetative cover can be used as a self-sustaining cap to control erosion and reduce water drainage (Meers et al., 2007). Dense short-rotation coppicing limits spread of pollutants thanks to its water uptake and canopy interception of precipitation (Perttu, 1998). Several studies have also demonstrated that many woody species and clones of *Salix*, *Populus* and *Alnus* have the ability to accumulate appreciable amounts of metals in above-ground biomasses (Rosselli et al., 2003; Laurysens et al., 2004; French et al., 2006; Bedell et al., 2009). These species are the climax vegetation on freshwater tidal marshes and other sediment-derived substrates contaminated with metals (Vandecasteele et al., 2006). Appropriate management practices for these types of land must aim at minimising the risks of contaminant transfer. Recent research has focused on proper choice of tree species and the use of thin capping layers as management options for markedly polluted sediments, and on the effects of metals on litter decomposition (Mertens et al., 2007; Vandecasteele et al., 2005a, 2008).

The hydrological conditions of polluted sites can greatly affect the bioavailable fraction of trace elements. For instance, an upland hydrological regime resulted in elevated Cd and Zn concentrations in the leaves of *Salix cinerea*, whereas concentrations were normal when pots were kept at a wetland hydrological regime (Vandecasteele et al., 2005c).

In this study, metal concentrations in various species were investigated in a contaminated canal sediment in north-western England (UK). As the canal had been closed to shipping in the recent past and its water level had progressively decreased, there is the need to identify a suitable vegetation cover to stabilise the sediment. This study investigating the phytoremediation potential of species (leaves and wood) planted in the dredged sediment six years previously, in comparison with spontaneous vegetation. Suitable inorganic amendments, including cement, lime and iron sulphate, were examined in the laboratory in order to identify suitable means for reduce metal leaching in the alternate conditions of sub-immersion.

Materials and methods

Site description

The trial was carried out on sediments dredged from the Woolston New Cut Canal in NW England (UK) (53° 23'40'' N, 2° 33'29'' W). The canal was cut in 1821 to better connect the industrial cities of Liverpool and Manchester through the Mersey river, and has an average width of 15 m. The sediment became contaminated with heavy metals and organic pollutants from various industries and shipping (King et al., 2006). Since the canal was closed to navigation and disconnected from the Mersey in 1978, the water level has progressively fallen. Although the canal bed is gradually drying out, it still remains submerged for most of the year (Hartley and Dickinson, 2009).

The sediment is silty loam, with negligible calcium carbonate content, high organic matter and a low oxidised pH (Table 1). Total concentrations of As, Cd, Cr, Cu, Pb and Zn exceeded the UK CLEA (Contaminated Land Exposure Assessment) soil guideline values (SGVs) and former ICRL (Interdepartmental Committee on the Reclamation of Contaminated Land) guidelines for soils in areas of recreational land use (King et al., 2006). The exposed sediment has been colonised by spontaneous vegetation, such as gray willow (*Salix atrocinerea* Brot.), bulrush (*Typha latifolia* L.) and common reed (*Phragmites australis* (Cav.) Steud.).

Table 1. Physico-chemical parameters of sediment, including significantly elevated pseudo-total trace element concentrations, at 0.8-1.1 m depth interval (From: Hartley and Dickinson, 2009).

Parameter	Value
pH (wet sediment)	6.55
pH (dry sediment)*	3.67
pH (canal water)	6.39
Sediment water content	75%
Sand	29.3%
Silt	65.2%
Clay	5.48%
LOI	26.7%
Total Carbon	15.2%
Total Sulphate (mg kg ⁻¹ , dry sediment)	7184
Total Iron (mg kg ⁻¹)	5,940
Total Phosphate (mg kg ⁻¹)	4,280
CaCO ₃ **	<0.01%
As (mg kg ⁻¹)	145
Cd (mg kg ⁻¹)**	20
Cr (mg kg ⁻¹)	1,315
Cu (mg kg ⁻¹)	887
Ni (mg kg ⁻¹)	102
Pb (mg kg ⁻¹)	1,887
Zn (mg kg ⁻¹)	4,858

* Sediment was air-dried and water was added to make a slurry.

** Values from King et al. (2006).

Analyses of spontaneous vegetation

Samples of spontaneous species, leaves in *Typha latifolia* and *Phragmites australis*, and leaves and twigs in *Salix atrocinerea*, were collected and washed with deionised water before oven-drying at 45°C. About 0.2 g of dried and ground biomass was added with 10 mL of Analar grade 15.55 M HNO₃ and digested in a microwave oven (CEM Mars Xpress microwave). Solutions were analysed by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS, XSERIES 2; Thermo Scientific, MA, USA) together with certified reference material.

In order to provide an overall ability to accumulate various metals, concentrations were standardised (divided) by the hyperaccumulation thresholds reported in the literature, i.e., 100 mg kg⁻¹ for Cd, 1,000 mg kg⁻¹ for As, Cr, Cu, Ni, and Pb, and 10,000 mg kg⁻¹ for Cr and Zn (Terry and Bañuelos, 2000; Visoottiviseth et al., 2002).

The metal biological absorption coefficient (BAC), i.e., the plant (harvestable)-to-sediment metal concentration ratio (Blaylock et al., 1997) was calculated for individual metals and their summation.

Analyses of cultivated dredge sediments

In 2002, spontaneous vegetation was cleared from a 150-m section of the canal. Sediment was dredged and deposited on the canal basin (150 m length × 3 m breadth × 0.5 m depth), which was subdivided into experimental plots. Twelve woody *taxa* were planted at a density of 2.67 plants m⁻², in a randomised block design with six replicates (Royle et al., 2003). The species were hybrids and clones of willow (*Salix*), poplar (*Populus*) and alder (*Alnus*) (Table 2) (King et al., 2006).

Table 2. Hybrids and clones of tree species planted in dredged canal sediment in May 2002 (King et al., 2006)

Species/clone (<i>taxa</i>)	Clone name
<i>Alnus cordata</i> Lois.	
<i>A. glutinosa</i> L.	
<i>A. incana</i> L.	
<i>Populus deltoids</i> × <i>nigra</i>	Ghoy
<i>P. trichocarpa</i>	Trichobel
<i>Salix atrocinerea</i> Brot.	
<i>S. caprea</i> × <i>cinerea</i> × <i>viminialis</i>	Calodendron
<i>S. fragilis</i> L.	
<i>S. viminalis</i> L.	Jorunn
<i>S. viminalis</i> × <i>burjatica</i>	Ashton Stott
<i>S. viminalis</i> × <i>caprea</i>	Sericans
<i>S. viminalis</i> × <i>schwerinn</i>	Tora

In 2008, plants from one block were sampled, leaves and wood (older branches) separately, washed with deionised water and oven-dried at 45° C. According to the method described above for spontaneous vegetation, samples were digested and analysed by ICP-MS.

Soil was sampled in the first 0.15 m of five randomly chosen plots, planted with *Alnus cordata* Lois., *A. incana* L., *Populus trichocarpa* ‘Trichobel’, *S. viminalis* × *caprea* ‘Sericans’, and *S. viminalis* × *schwerinn* ‘Tora’. Samples were crushed in a mortar and sieved (<2 mm). Acid digestion and ICP-MS analysis followed were the same procedure as that described for vegetation analysis.

Total organic carbon was determined on 0.03 g of soil in a TOC-VCSN Shimadzu analyzer (Shimadzu, Tokyo, Japan).

For cultivated species, the overall capacity to concentrate above-ground pollutants was evaluated through summation of moles of 7 sediment-polluting trace elements.

The metal biological absorption coefficient (BAC), i.e., the plant (harvestable)-to-sediment metal concentration ratio for individual metals and their summation, in *Alnus cordata*, *Alnus incana*, *Populus* ‘Trichobel’, *Salix* ‘Sericans’ and *Salix* ‘Tora’, was calculated by considering the mean values of leaves and wood.

Sediment stabilisation

The possibility of immobilising some of the pollutants in the sediment (Cd, Cu, Pb, Zn) was explored by amending air-dried, 2-mm sieved canal sediment with Portland cement (1, 20, and 50% w/w), lime (CaCO₃, 1% w/w), and lime and iron (III) sulphate pentahydrate (97% Fe₂(SO₄)₃·5H₂O) (both 0.5% w/w). The cement contained about 67% of CaO, 23% SiO₂, 5.5% Al₂O₃, and 4.5% S. The horticultural-grade lime used was a commercial formulation with a neutralising value of 45% (total passing through a 150-µm sieve: 20%).

In order to mimic canal conditions, where sediment was cyclically exposed to flooding and drying, four cycles of wet-and-dry extraction were performed. About 1 g of pure (untreated controls) or amended sediment was added with 15 mL of deionised water and shaken at 200 oscillations per minute for 24 hours. After centrifugation at 3,000 rpm,

the supernatant was collected, filtered through Whatman fibreless filter paper, and added with a drop of HNO₃. Each solution was analysed in triplicate by Atomic Absorption Spectroscopy (AAS) for determination of Cd, Cu, Pb, and Zn contents. Pellets were oven-dried at 45° C for 24 hours and then resuspended in water for the following cycle.

Statistical analysis

Statistical analysis was carried out by Statgraphics software (Manugistic Inc., Rockville, MD, USA). ANOVA and the LSD test ($P \leq 0.05$) were used to evaluate differences among means, after checking normality of data.

Results

Metal accumulation in vegetation

Metal concentration in the above-ground biomass was fairly similar among species of the spontaneous flora grown in dredged sediments, as confirmed by statistical analysis (Table 3). Only *Salix atrocinerea* showed higher Zn together with better BAC (1.4% vs. 0.53 and 0.46 of bulrush and common reed, respectively).

For cultivated plants, high organic carbon (TOC) was measured in the plots of *Alnus incana* (28.5%) and *Populus* ‘Trichobel’ (22.4%). Instead, *Salix* ‘Sericans’, *Salix* ‘Tora’ and *Alnus cordata* had lower TOC in their plots (12.7, 12.3 and 11.9%, respectively).

Cd and Cu mostly polluted the plot of *Alnus cordata*, whereas the sediment of *A. incana* was highly contaminated by Ni and Zn. Sediment Cr and Ni were higher for *Populus* ‘Trichobel’, whereas As, Cr and Pb were higher in *Salix* ‘Sericans’ (Table 4).

Table 3. Individual metal concentrations and their summation ($\Sigma[\text{HM}_{\text{st}}]$) (standardised values) in spontaneous species ($\pm\text{S.E.}$, $n=3$) (above-ground biomass and *Salix* twigs). Letters: comparisons among species for same metal.

Metal	<i>Phragmites australis</i>		<i>Typha latifolia</i>		<i>Salix atrocinerea</i>	
As	0.13 $\pm$ 0.01	a	0.11 $\pm$ 0.00	a	0.10 $\pm$ 0.02	a
Cd	0.88 $\pm$ 0.39	a	0.24 $\pm$ 0.05	a	0.99 $\pm$ 0.43	a
Cr	7.10 $\pm$ 0.19	a	6.86 $\pm$ 0.08	a	6.96 $\pm$ 0.47	a
Cu	6.34 $\pm$ 2.24	a	3.06 $\pm$ 0.10	a	6.00 $\pm$ 0.66	a
Ni	7.59 $\pm$ 0.90	a	8.57 $\pm$ 1.53	a	8.18 $\pm$ 1.18	a
Pb	6.46 $\pm$ 0.81	a	8.12 $\pm$ 2.09	a	6.18 $\pm$ 1.41	a
Zn	26.0 $\pm$ 6.34	b	22.8 $\pm$ 3.70	b	68.5 $\pm$ 6.36	a
$\Sigma[\text{HM}_{\text{st}}]$	0.03 $\pm$ 0.01	a	0.03 $\pm$ 0.00	a	0.04 $\pm$ 0.01	a

Table 4. Metal concentrations ($\pm$ S.E., n=3) in sediments of plots/cultivated species. Letters: statistically significant differences among plots/species (LSD Test, $P \leq 0.05$).

Plot/Species	As	Cd	Cr	Cu	Ni	Pb	Zn
<i>Alnus cordata</i>	206 $\pm$ 8.58 c	9.16 $\pm$ 0.36 a	676 $\pm$ 3.87 b	410 $\pm$ 11.3 a	29.9 $\pm$ 0.67 b	981 $\pm$ 11.3 c	472 $\pm$ 16.9 b
<i>Alnus incana</i>	44.1 $\pm$ 2.46 e	3.70 $\pm$ 0.26 b	401 $\pm$ 20.6 c	251 $\pm$ 11.6 c	36.8 $\pm$ 2.51 a	692 $\pm$ 28.6 d	628 $\pm$ 31.1 a
<i>Populus</i> . 'Trichobel'	104 $\pm$ 1.76 d	1.35 $\pm$ 0.25 d	797 $\pm$ 22.7 a	341 $\pm$ 3.15 b	33.4 $\pm$ 1.25 ab	1,122 $\pm$ 8.87 b	170 $\pm$ 2.62 d
<i>Salix</i> 'Sericans'	404 $\pm$ 10.6 a	1.26 $\pm$ 0.28 d	786 $\pm$ 26.6 a	272 $\pm$ 5.32 c	25.4 $\pm$ 0.99 c	1,264 $\pm$ 13.0 a	132 $\pm$ 2.98 d
<i>Salix</i> 'Tora'	275 $\pm$ 4.36 b	2.65 $\pm$ 0.11 c	634 $\pm$ 16.5 b	350 $\pm$ 8.46 b	30.5 $\pm$ 0.53 b	969 $\pm$ 28.0 c	383 $\pm$ 9.00 c
MEAN	207 $\pm$ 34.0	3.62 $\pm$ 0.78	659 $\pm$ 38.9	325 $\pm$ 15.6	31.2 $\pm$ 1.14	1,006 $\pm$ 51.3	357 $\pm$ 50.1
C.V. %	64	84	23	19	14	20	54

Table 5. Metal concentration (mg kg⁻¹, $\pm$ S.E., mg kg⁻¹, n=3) in plant compartments (leaves, wood) of trees cultivated on dredged sediments. Small letters: comparison among species within same organ and metal. Capital letters: comparisons among organs for same metal. LSD Test, P $\leq$ 0.05.

Species	As	Cd	Cr	Cu	Ni	Pb	Zn
Leaves							
<i>Alnus cordata</i>	0.16 $\pm$ 0.02 cd	0.66 $\pm$ 0.16 e	3.15 $\pm$ 1.56 n.s.	10.6 $\pm$ 2.36 ab	10.9 $\pm$ 1.63 n.s.	9.44 $\pm$ 2.19 b	278 $\pm$ 59.8 ef
<i>A. glutinosa</i>	0.15 $\pm$ 0.03 cd	0.56 $\pm$ 0.05 e	3.59 $\pm$ 1.88 n.s.	13.6 $\pm$ 2.16 a	9.50 $\pm$ 2.45 n.s.	16.6 $\pm$ 6.17 ab	199 $\pm$ 17.4 f
<i>A. incana</i>	0.15 $\pm$ 0.06 cd	0.60 $\pm$ 0.19 e	3.15 $\pm$ 1.59 n.s.	9.85 $\pm$ 0.61 ab	10.2 $\pm$ 1.11 n.s.	6.16 $\pm$ 4.12 b	150 $\pm$ 20.3 f
<i>Populus</i> ‘Ghoy’	0.45 $\pm$ 0.05 a	2.19 $\pm$ 0.15 cd	3.46 $\pm$ 1.93 n.s.	5.06 $\pm$ 0.58 c	8.58 $\pm$ 1.57 n.s.	3.14 $\pm$ 0.69 b	759 $\pm$ 50.4 b
<i>P.</i> ‘Trichobel’	0.19 $\pm$ 0.04 bcd	1.18 $\pm$ 0.08 de	3.46 $\pm$ 1.88 n.s.	6.87 $\pm$ 1.17 bc	11.1 $\pm$ 2.35 n.s.	3.95 $\pm$ 0.68 b	359 $\pm$ 82.9 def
<i>Salix</i> ‘Ashton Stott’	0.16 $\pm$ 0.04 cd	2.24 $\pm$ 0.22 cd	3.31 $\pm$ 1.76 n.s.	6.89 $\pm$ 2.47 bc	9.26 $\pm$ 1.99 n.s.	5.00 $\pm$ 2.16 b	332 $\pm$ 75.1 def
<i>S. atrocinerea</i>	0.06 $\pm$ 0.04 d	10.8 $\pm$ 1.28 a	3.70 $\pm$ 1.82 n.s.	6.59 $\pm$ 1.38 bc	13.4 $\pm$ 3.54 n.s.	4.45 $\pm$ 1.08 b	1042 $\pm$ 125 a
<i>S.</i> ‘Calodendron’	0.40 $\pm$ 0.09 ab	1.81 $\pm$ 0.31 cde	4.62 $\pm$ 2.22 n.s.	7.83 $\pm$ 1.23 bc	7.37 $\pm$ 1.64 n.s.	37.3 $\pm$ 32.2 a	227 $\pm$ 45.3 ef
<i>S. fragilis</i>	0.29 $\pm$ 0.12 abc	1.22 $\pm$ 0.09 de	2.53 $\pm$ 1.35 n.s.	6.68 $\pm$ 0.84 bc	10.6 $\pm$ 1.88 n.s.	2.55 $\pm$ 0.99 b	455 $\pm$ 46.1 cde
<i>S.</i> ‘Jorunn’	0.29 $\pm$ 0.07 abc	2.49 $\pm$ 0.29 cd	3.54 $\pm$ 1.92 n.s.	6.26 $\pm$ 1.30 bc	10.9 $\pm$ 2.64 n.s.	16.2 $\pm$ 13.5 ab	509 $\pm$ 34.4 cd
<i>S.</i> ‘Sericans’	0.40 $\pm$ 0.11 a	2.81 $\pm$ 0.79 c	3.84 $\pm$ 1.90 n.s.	8.26 $\pm$ 2.68 bc	9.86 $\pm$ 2.21 n.s.	4.60 $\pm$ 2.08 b	627 $\pm$ 92.2 bc
<i>S.</i> ‘Tora’	0.27 $\pm$ 0.11 abcd	4.48 $\pm$ 0.54 b	2.87 $\pm$ 1.52 n.s.	5.17 $\pm$ 0.45 c	12.5 $\pm$ 2.12 n.s.	2.70 $\pm$ 0.64 b	788 $\pm$ 127 b
Mean	0.24 $\pm$ 0.03 A	2.60 $\pm$ 0.42 A	3.41 $\pm$ 0.45 A	7.80 $\pm$ 0.54 A	10.4 $\pm$ 0.60 A	8.74 $\pm$ 2.44 A	482 $\pm$ 43.4 A
Wood							
<i>Alnus cordata</i>	0.07 $\pm$ 0.02 e	0.66 $\pm$ 0.09 f	1.44 $\pm$ 1.16 n.s.	6.42 $\pm$ 1.26 bc	5.14 $\pm$ 0.47 abc	8.18 $\pm$ 1.60 ab	130 $\pm$ 18.7 cd
<i>A. glutinosa</i>	0.09 $\pm$ 0.03 cde	0.74 $\pm$ 0.18 f	2.63 $\pm$ 1.35 n.s.	8.52 $\pm$ 2.71 bc	5.74 $\pm$ 0.67 ab	13.9 $\pm$ 1.85 ab	113 $\pm$ 20.6 cd
<i>A. incana</i>	0.07 $\pm$ 0.03 de	0.85 $\pm$ 0.39 ef	2.46 $\pm$ 1.24 n.s.	6.66 $\pm$ 2.54 bc	4.85 $\pm$ 1.33 abc	4.29 $\pm$ 1.34 b	75 $\pm$ 7.74 d
<i>Populus</i> ‘Ghoy’	0.19 $\pm$ 0.03 ab	1.53 $\pm$ 0.25 cde	2.68 $\pm$ 1.40 n.s.	2.49 $\pm$ 0.51 c	5.38 $\pm$ 1.73 abc	4.25 $\pm$ 1.11 b	187 $\pm$ 42.4 bc
<i>P.</i> ‘Trichobel’	0.12 $\pm$ 0.03 bcde	0.77 $\pm$ 0.13 f	1.41 $\pm$ 1.11 n.s.	8.06 $\pm$ 2.72 bc	5.08 $\pm$ 0.54 abc	4.78 $\pm$ 0.83 b	82 $\pm$ 20.5 d
<i>Salix</i> ‘Ashton Stott’	0.14 $\pm$ 0.04 abcde	2.11 $\pm$ 0.20 c	2.16 $\pm$ 1.22 n.s.	8.80 $\pm$ 3.84 bc	6.32 $\pm$ 2.08 a	7.07 $\pm$ 2.91 b	232 $\pm$ 57.6 b
<i>S. atrocinerea</i>	0.12 $\pm$ 0.04 bcde	6.99 $\pm$ 0.59 a	2.41 $\pm$ 1.25 n.s.	6.63 $\pm$ 1.10 bc	5.46 $\pm$ 0.71 abc	9.45 $\pm$ 2.89 ab	379 $\pm$ 65.9 a
<i>S.</i> ‘Calodendron’	0.22 $\pm$ 0.07 a	1.54 $\pm$ 0.17 cde	3.16 $\pm$ 1.57 n.s.	34.9 $\pm$ 15.31 a	6.19 $\pm$ 1.65 ab	13.0 $\pm$ 8.06 ab	122 $\pm$ 25.2 cd
<i>S. fragilis</i>	0.22 $\pm$ 0.01 a	1.20 $\pm$ 0.37 def	2.55 $\pm$ 1.28 n.s.	15.4 $\pm$ 2.28 b	3.69 $\pm$ 0.25 bc	23.3 $\pm$ 19.7 a	97 $\pm$ 12.1 cd
<i>S.</i> ‘Jorunn’	0.17 $\pm$ 0.04 abc	1.63 $\pm$ 0.09 cd	1.41 $\pm$ 1.08 n.s.	7.13 $\pm$ 2.67 bc	3.15 $\pm$ 0.43 c	6.51 $\pm$ 1.87 b	232 $\pm$ 40.2 b
<i>S.</i> ‘Sericans’	0.15 $\pm$ 0.03 abcd	2.04 $\pm$ 0.18 c	1.46 $\pm$ 0.89 n.s.	5.94 $\pm$ 1.92 bc	4.48 $\pm$ 0.57 abc	5.87 $\pm$ 2.89 b	243 $\pm$ 11.3 b
<i>S.</i> ‘Tora’	0.13 $\pm$ 0.02 bcde	2.89 $\pm$ 0.48 b	2.70 $\pm$ 1.36 n.s.	7.18 $\pm$ 2.47 bc	5.29 $\pm$ 0.54 abc	5.29 $\pm$ 1.86 b	254 $\pm$ 78.4 b
Mean	0.14 $\pm$ 0.01 B	1.91 $\pm$ 0.25 B	2.21 $\pm$ 0.33 A	9.84 $\pm$ 1.72 A	5.06 $\pm$ 0.30 B	8.83 $\pm$ 1.80 A	179 $\pm$ 16.3 B

Table 6. Metal concentrations in aboveground biomass (mean of leaves and wood) (mg kg⁻¹, $\pm$ S.E., n=3) and coefficient of variation for *taxa* of same species in dredged sediments. Small letters: differences among species for same metal (LSD Test, P $\leq$ 0.05).

Species	As	Cd	Cr	Cu	Ni	Pb	Zn
<i>Alnus cordata</i>	0.12 $\pm$ 0.02 e	0.66 $\pm$ 0.08 f	2.29 $\pm$ 0.96 n.s.	8.49 $\pm$ 1.46 b	8.01 $\pm$ 1.34 n.s.	8.81 $\pm$ 1.28 ab	204 $\pm$ 40.4 ef
<i>A. glutinosa</i>	0.12 $\pm$ 0.02 de	0.65 $\pm$ 0.09 f	3.11 $\pm$ 1.09 n.s.	11.1 $\pm$ 1.87 b	7.62 $\pm$ 1.37 n.s.	15.3 $\pm$ 3.02 ab	156 $\pm$ 20.5 f
<i>A. incana</i>	0.11 $\pm$ 0.04 e	0.72 $\pm$ 0.21 f	2.80 $\pm$ 0.95 n.s.	8.26 $\pm$ 1.35 b	7.52 $\pm$ 1.29 n.s.	5.22 $\pm$ 2.03 b	112 $\pm$ 17.4 f
<i>Populus</i> 'Ghoy'	0.32 $\pm$ 0.06 a	1.86 $\pm$ 0.18 cd	3.07 $\pm$ 1.11 n.s.	3.78 $\pm$ 0.60 b	6.98 $\pm$ 1.24 n.s.	3.70 $\pm$ 0.64 b	473 $\pm$ 112 bc
<i>P.</i> 'Trichobel'	0.16 $\pm$ 0.03 cde	0.98 $\pm$ 0.10 ef	2.43 $\pm$ 1.08 n.s.	7.47 $\pm$ 1.39 b	8.10 $\pm$ 1.60 n.s.	4.36 $\pm$ 0.52 b	221 $\pm$ 65.5 ef
<i>Salix</i> 'Ashton Stott'	0.15 $\pm$ 0.03 cde	2.17 $\pm$ 0.14 cd	2.73 $\pm$ 1.02 n.s.	7.85 $\pm$ 2.15 b	7.79 $\pm$ 1.44 n.s.	6.03 $\pm$ 1.72 b	282 $\pm$ 47.7 de
<i>S. atrocinerea</i>	0.09 $\pm$ 0.03 e	8.90 $\pm$ 0.97 a	3.06 $\pm$ 1.05 n.s.	6.61 $\pm$ 0.82 b	9.46 $\pm$ 2.25 n.s.	6.95 $\pm$ 1.71 b	711 $\pm$ 141 a
<i>S.</i> 'Calodendron'	0.29 $\pm$ 0.06 ab	1.66 $\pm$ 0.16 cde	3.79 $\pm$ 1.23 n.s.	23.3 $\pm$ 9.85 a	6.70 $\pm$ 1.10 n.s.	23.4 $\pm$ 13.8 ab	167 $\pm$ 30.4 ef
<i>S. fragilis</i>	0.25 $\pm$ 0.06 abc	1.21 $\pm$ 0.18 def	2.54 $\pm$ 0.86 n.s.	11.0 $\pm$ 1.99 b	7.17 $\pm$ 1.58 n.s.	12.9 $\pm$ 9.96 ab	276 $\pm$ 71.1 de
<i>S.</i> 'Jorunn'	0.23 $\pm$ 0.04 abcd	2.06 $\pm$ 0.21 cd	2.47 $\pm$ 1.09 n.s.	6.69 $\pm$ 1.39 b	7.05 $\pm$ 1.92 n.s.	11.4 $\pm$ 6.58 ab	371 $\pm$ 57.8 cd
<i>S.</i> 'Sericans'	0.28 $\pm$ 0.07 ab	2.42 $\pm$ 0.40 cd	2.65 $\pm$ 1.07 n.s.	7.10 $\pm$ 1.59 b	7.17 $\pm$ 1.47 n.s.	5.23 $\pm$ 1.67 b	435 $\pm$ 84.3 bc
<i>S.</i> 'Tora'	0.20 $\pm$ 0.06 bcde	3.69 $\pm$ 0.45 b	2.79 $\pm$ 0.95 n.s.	6.17 $\pm$ 1.22 b	8.88 $\pm$ 1.69 n.s.	3.99 $\pm$ 1.03 b	521 $\pm$ 122 bc
MEAN (<i>Alnus</i>)	0.12 $\pm$ 0.02	0.68 $\pm$ 0.08	2.74 $\pm$ 0.56	9.27 $\pm$ 0.91	7.72 $\pm$ 0.74	9.76 $\pm$ 1.50	157 $\pm$ 17.3
MEAN % (<i>Populus</i>)	0.24 $\pm$ 0.04	1.42 $\pm$ 0.15	2.75 $\pm$ 0.75	9.62 $\pm$ 0.87	7.54 $\pm$ 0.99	4.03 $\pm$ 0.41	347 $\pm$ 70.8
MEAN % (<i>Salix</i>)	0.21 $\pm$ 0.02	3.19 $\pm$ 0.37	2.84 $\pm$ 0.37	9.57 $\pm$ 1.49	7.76 $\pm$ 0.63	9.74 $\pm$ 2.50	399 $\pm$ 39.0
C.V. % (<i>Alnus</i>)	3	6	15	17	3	52	29
C.V. % (<i>Populus</i>)	49	44	16	46	11	12	52
C.V. % (<i>Salix</i>)	34	84	16	63	13	68	46

Metal concentrations in the above-ground biomass of twelve species and clones revealed a higher accumulation of various trace elements such as As, Cd, Ni and Zn in leaves than in woody tissues (Table 5). Arsenic was generally low and better accumulated by *Populus* ‘Ghoy’ (0.32 mg kg⁻¹) and some *Salix*, regardless of plant organ. Cd and Zn reached more interesting concentrations in *Salix atrocinerea* (8.90 and 711 mg kg⁻¹, respectively), and Cu (23.3 mg kg⁻¹) and Pb (23.4 mg kg⁻¹) in *Salix* ‘Calodendron’. For Cu and Ni, differences among species were marginal (Table 6).

In general, the metal most frequently taken up was Zn, accounting for more than 90% of removals, followed by Cu and Ni (3.6% and 3%, respectively). The coefficients of variation for metal concentrations were more restricted in *Alnus* than in the other species, ranging from 3% for As and Ni to 52% for Pb. Coefficients of variation observed in *Populus* and *Salix* species were much higher, especially for Cd (84%) and Pb (68%) (*Salix*). In both wood and leaves, the overall metal concentrations (7 trace elements combined) were clearly highest in *Salix atrocinerea*, followed by *S.* ‘Tora’ (Fig. 1).

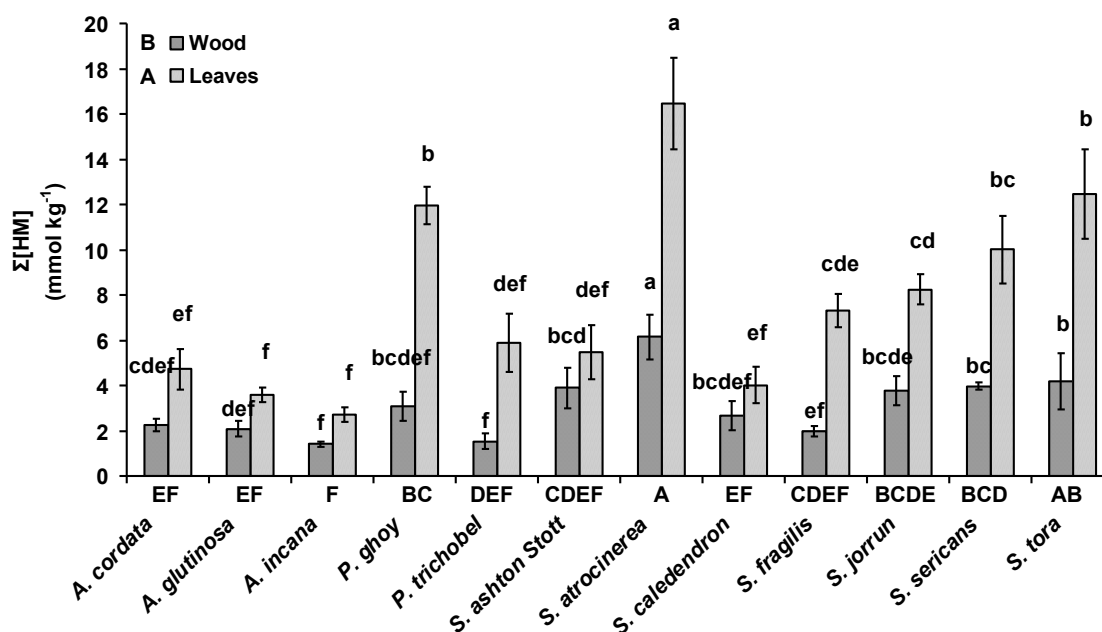


Figure 1. Summation of concentrations (mmol kg⁻¹ d.w., ± S.E., n=3) in leaves and wood of 7 trace elements (As, Cd, Cr, Cu, Ni, Pb, Zn). Capital letters: differences among species or plant organ (main effects). Small letters: comparisons among species within same organ. LSD Test, P≤0.05.

The phytoextraction ability of *Alnus* spp. was generally poor. Like the spontaneous flora, the BAC was generally small, regardless of species choice. Values were: 2.4% (Cu), 0.42% (Cr), 0.17% (Pb) and 0.12% (As). The Cd and Zn BAC were unexpectedly high, with 225% and 131%, respectively, followed by Ni (26%). Among species, *Salix* 'Sericans' had the most efficient BAC, followed by *Populus* 'Trichobel' and *S.* 'Tora'.

Sediment stabilisation

In untreated sediments, Pb losses were very high. Leaching of Cd in controls was higher in the second wetting/drying cycle, whereas Cu and Pb were most leached during the third (Cu) and fourth (Cu and Pb) (Fig. 2). Conversely, most Zn entered solution during the first cycle.

A low dose of cement (1%) did not affect Cd and Cu mobility, but was able to reduce significantly the amounts of solubilised Pb (-53%) and Zn (-87%). Higher doses of cement (20% and 50%) again reduced Pb (-91%) and Zn (-99%) mobility, irrespective of the dose applied, but Cu was, unexpectedly, extensively leached (+208%).

Lime had a positive effect on the stabilisation of Pb (-65%), Cu (-46%) and Zn (-22%), but not of Cd. With lime, Pb and Cu were mainly immobilised in the first cycle and much less in the last fourth cycle, unlike Zn.

The addition to sediments of iron sulphate together with lime had the same positive effect of lime alone for Cu and Pb mobility, but greatly increased leaching of Cd (2.5×) and Zn (1.9×), especially during the first two cycles.

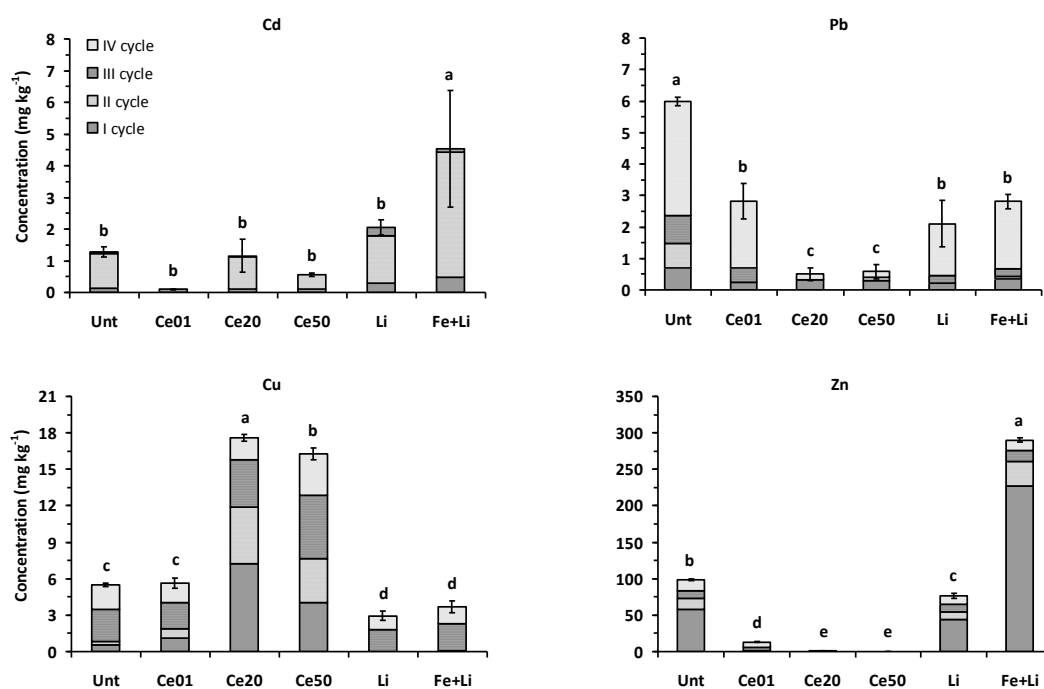


Figure 2. Metal leaching during four cycles of wetting and drying extraction as a function of sediment treatments [untreated (Unt); cement 1% w/w (Ce01); cement 20% w/w (Ce20); cement 50% w/w (Ce50); lime 1% w/w (Li); iron sulphate 0.5% and lime 0.5% w/w (Fe+Li)]. Results are expressed as mg of leached metal per kg of sediment (n=3). Bars: standard errors for total leaching. Letters: differences among total leaching (LSD Test, $P \leq 0.05$).

Discussion

The Woolston New Cut Canal dredge sediment was an inhospitable substrate for plant growth, especially in the first period after transplant (King et al., 2006). Visual evaluation of the vegetation after a reasonably long period (six years), revealed that it had reached good establishment and cover.

The physico-chemical characteristics of the sediment have evolved in time. After initial intensely oxidised pH reduction of dredged sediments, which led to increased metal mobility and phytotoxicity, pollution was probably mitigated in time.

Metals were heterogeneously distributed within the sediment, as highlighted by marked changes across close plots. However, variations in metal concentrations were not generally reflected in plant accumulation, with only a few exceptions – As, Cu and Pb. This may be explained by the attainment of a new equilibrium between total and bioavailable metals, rather than by restricted variation of species.

Metal concentrations in the above-ground biomass of woody clones and species were often limited and several times lower than the hyperaccumulation thresholds (Terry and Bañuelos, 2000; Visoottiviseth et al., 2002), but the BAC was seldom appreciable, as found for Cd and Zn. The great inter- and intra-specific variability for metal accumulation makes species selection an essential key to the phytoremediation of these sediments. Results were poor for *Alnus*, whereas *Populus* and *Salix* showed good accumulations and large variability, which deserve to be exploited. In the literature, metal accumulation by *Fraxinus excelsior* L., *Alnus glutinosa* L. Gaertn., *Acer pseudoplatanus* L. and *Robinia pseudoacacia* L., is also found stable in contaminated and control environments (Mertens et al., 2004). Instead, good performance was achieved for Cd, Cu and Zn in *Populus alba* L. (Mertens et al., 2004; Vamerali et al., 2009) and *Salix cinerea* L. (Vandecasteele et al., 2005b), corroborating our results.

For various metals, the green tissues (leaves) showed greater concentrations than in wood – a result also found by Mosca et al. (2004) in a pluricontaminated soil. This response was particularly evident for Cd (Robinson et al., 2000; Klang-Westin and Eriksson, 2003), which reached concentrations in willow and poplar leaves 1-5 times greater than in wood. Although the woody biomass production found by the above authors was approximately double that of litter deposition, mass removal of metals was still better with wood.

In oxidised sediments, the reduced efficiency of trees in metal removals is expected in the long term, as highlighted by Tack and Vandecasteele (2009), who found worsening with stand age, due to higher metal accumulation in actively growing tissues such as shoots and young leaves. High transfers to leaf tissues of zootoxic metals like As, Cd, Ni and Zn raises the problem of metal recycling through vegetation and increases the risks for

herbivores. Further interferences with the surrounding environment are expected through decomposing litter, an increase of metal concentrations being expected if output fluxes exceed input ones. In our case, only Cd, Zn and occasionally Ni had high BAC in willow and poplar species – an occurrence which should be carefully evaluated. Recent results in the literature (Vandecasteele et al., 2009) do confirm significant Cd and Zn increases in the top-soil of infrastructure spoil landfills created more than 20 years ago and mainly colonised by willow. Mertens et al. (2007) also reports increasing organic matter and Cd and Zn concentrations in the upper 5-cm layer of soil colonised by poplar after more than 30 years of afforestation, an occurrence which can also retard litter decomposition (Berg et al., 2003).

Attempts at stabilising sediments by amendment have produced variable effects, depending on the metal in question and the type of amendment. Cement has the purpose of forming a cap to prevent metal losses, and lime is used to raise sediment pH. Since lime alone is liable to As leaching, the contemporary addition of iron sulphate aims at offsetting this effect, as it is a good immobiliser of As (Hartley et al., 2004).

It has been established that Cu in sediments is bound with the organic matter and sulphide fractions (Hartley and Dickinson, 2009) and, although we confirm the reduction of Cu mobility with lime and lime+iron sulphide, great attention should be paid in managing high percentages of cement. The negative effect of high cement doses on Cu may be a consequence of a change in pH and possible oxidation of organic matter.

The effects of lime on Cd and that of 1% cement on Cu were both negligible, but the application of both is feasible for the other metals. The relatively small activity rate of cement and lime enhances their cheap application on large scales, but care should be taken for lime, due to pH increases and the associated possible mobilisation of As (Hartley et al., 2004). Any attempt at mitigating this effect by the addition of iron sulphate is not recommended, as it causes excessively high risks of Cd and Zn mobilisation, as previously found by Hartley et al. (2004).

Conclusions

Continuous wetting and drying of sediments frequently cause profound changes in sediment conditions and chemistry, increasing metal and arsenic mobility. There are obvious difficulties in managing contamination by both arsenic and metals, because of their opposite responses to chemical soil variations. In the Cut Canal sediment, great concern arose due to high mobility of Pb, which was higher in controls than in amended soil. However, the addition of small doses of cement or lime is a cheap and efficient way of stabilising most cation metals, with the exception of copper (cement) and cadmium (lime). The high root affinity for copper found in *Populus* and *Salix* species (Vamerali et al., 2009) suggests the opportunity of combining profitably soil amendment with the cultivation of selected species. Although in a previous study on the same sediments (King et al., 2006) phytoextraction and phytostabilisation did not seem feasible management options, due to high plant mortality and low metal uptake, the site now has a well-established vegetation cover. However, phytoremediation is still largely not feasible, with the exception of Zn and Cd. The leaves of a large number of species tested contained higher metal concentrations than their wood, and harvesting them would stop metals re-entering the sediment. This option is difficult to pursue, but the leaf biomass of adult deciduous plants may account for even half the annual production of biomass. More precise quantifications of yields in polyannual species and metal distributions within plant organs would provide better insights to the management of these contaminated sediments.

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General conclusions

Plant acquisition of the most mobile metal fraction in soil, a technique known as *Bioavailable Contaminant Stripping*, is rarely an efficient phytomanagement option in severely contaminated media like pyrite wastes. The only exception regards Zn and occasionally Cu, by means of appropriate species selection. Choice of suitable agronomic practices and adjustment of the cultivation method of biomass species appear to be essential ways of making phytomanagement of polluted sites more reliable. Exploration at pot level of the effects of auxins, humic acids, chelators and amendments can help the efficient transfer of results for *in situ* phytoremediation of metal-polluted media.

The use of biomass species as alternatives to hyperaccumulators, which generally fails in pluricontaminated sites, is currently an option under study, and both intra- and interspecific exploitation of variability in metal tolerance and accumulation is extremely useful. In this respect, the *Brassicaceae* family is one of the most interesting botanical families, and the great adaptability of fodder radish in pyrite wastes confirms previous results in a pluricontaminated site in Milan (Mosca et al., 2004), indicating the opportunity for its generalised cultivation in various conditions of contamination.

Most of the tools experimented here – auxins, humic acids, and EDDS – to increase metal uptake generally worsened above-ground productivity, depending on dosage, with reduced mass balances of phytoextraction. It is therefore necessary to identify precisely their appropriate dosages, improve soil fertility, and adjust plant density for wide-spaced crops.

For biomass species, increase in yields rather than in metal concentrations seems a more profitable strategy in phytoremediation. It also allows the vegetation cover to become established more efficiently, both for landscape purposes and for reduction of pollutant dispersion in the environment.

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