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Phytostabilization of heavy metals: role of plant roots and organic amendments

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Indice

Riassunto

L'argomento di questa tesi si colloca nell'ambito del "phytomanagement", cioè di quell'insieme di tecniche che vengono utilizzate per ridurre il contenuto o la mobilità dei metalli pesanti nei terreni grazie a processi mediati da piante superiori. Il "phytomanagement" comprende numerose tecniche che sono classificate in base al tipo di processo utilizzato e all'obbiettivo perseguito. In questo lavoro sono state approfondite le tecniche di fitostabilizzazione dei metalli pesanti, prendendone in considerazione entrambi gli aspetti di fitostabilizzazione *in-planta*, attraverso l'accumulo degli inquinanti nei tessuti radicali di specie da biomassa, e fitostabilizzazione *ex-planta*, attraverso l'impiego di ammendanti organici. Entrambe le tecniche hanno come obiettivo la riduzione della mobilità dei metalli tramite insolubilizzazione allo scopo di ridurne la disponibilità per gli organismi viventi, ma si distinguono per la localizzazione dei processi, rispettivamente nei tessuti della radice e nel suolo.

La sperimentazione sulla fitostabilizzazione *in-planta* è stata condotta con l'obiettivo di valutare la capacità di accumulo di metalli pesanti nelle radici fittonanti di una pianta modello (colza) e di definirne la dinamica di rilascio attraverso il processo di degradazione radicale. Sono stati valutati anche l'effetto di investimenti crescenti (22, 44 e 63 piante m^{-2}), del tipo varietale (due ibridi CHH a taglia convenzionale, un ibrido seminano e una varietà a impollinazione libera) (primo anno) e di un diverso livello di inquinamento da metalli nel terreno (secondo anno) sulla dinamica degradativa dei fittoni. I risultati indicano che, in un terreno non inquinato, la degradazione della biomassa radicale avviene abbastanza velocemente (-83% in un anno), anche se ~10% di materiale vegetale rimane indegradato dopo 18 mesi e in grado quindi di mantenere immobilizzati i metalli al suo interno; a questa sostanza organica recalcitrante, può tuttavia essere aggiunta annualmente o con il ciclo di coltivazione successivo nuova biomassa incrementando il pool organico per la ritenzione degli inquinanti. È stato evidenziato che i metalli vengono accumulati maggiormente nei tessuti radicali fibrosi (cortex interno) e il loro rilascio può risultare, in funzione dello specifico metallo, più lento della degradazione delle sostanza organica, con concentrazioni finali che variano a seconda dell'elemento. Il ritmo degradativo dei fittoni è risultato indipendente dal genotipo e dalla densità di

semina, ma sarebbero comunque da preferire cultivar più vigorose (ibridi CHH) e semine fitte in quanto garantirebbero una maggiore biomassa in campo (e.g., 1200 kg ha^{-1}per Taurus, che aumenta a 1700 kg ha^{-1} per investimenti di 63 piante m^{-2}) e un maggiore accumulo di metalli. L'inquinamento da metalli pesanti ha rallentato notevolmente la dinamica degradativa, a causa della minore attività microbica proteolitica (fasi iniziali) e cellulosolitica (fasi successive). La presenza di alte concentrazioni di metalli nel suolo ed elevati livelli di biodisponibilità (Cd, Co, Cu, Zn) può significativamente favorire l'adsorbimento degli stessi sul materiale organico in degradazione, determinando una dinamica temporale di accumulo nei fittoni in via di degradazione. Complessivamente, nonostante la degradazione della sostanza organica sia inevitabile, le radici fittonanti di una pianta annuale effettivamente consentono di stabilizzare i metalli nel lungo periodo, con livelli di efficienza maggiori nei terreni inquinati ai quali si rivolge questo tipo di tecnica.

Le prove di fitostabilizzazione *ex-planta* avevano come obbiettivo la valutazione del potenziale apporto di metalli pesanti ai suoli e sulla loro biodisponibilità in seguito a fertilizzazione con ammendanti organici derivati da materiali di scarto. È stato valutato anche il potenziale trasferimento di inquinanti alle piante coltivate. Sono stati confrontate diverse tipologie di ammendanti, evidenziando importanti effetti sul suolo e su sorgo da foraggio in funzione dalle caratteristiche dell'ammendante stesso, e in particolare dal suo grado di maturazione. A parità di C organico apportato, infatti, ammendanti che hanno subito processi di stabilizzazione (compostaggio) e che sono quindi più ricchi di nutrienti, e di azoto in particolare, ma anche di sostanze umiche, hanno fornito risultati produttivi migliori, favorendo nello stesso tempo l'accrescimento radicale. Nella sperimentazione sono stati confrontati compost da RSU, frazione solida di digestato da scarti vegetali e separato solido di liquame suino, ma in tutti i casi l'apporto di metalli pesanti al suolo è stato trascurabile così come l'accumulo nel foraggio del sorgo, indicando che per ammendanti prodotti a partire da materiali di qualità il rischio nella catena alimentare sembra limitato. Tuttavia, nel medio periodo, l'apporto di ammendanti organici può aumentare la biodisponibilità di alcuni elementi come Ni e Zn, indipendentemente dalla qualità della sostanza organica, anche se generalmente i rischi maggiori sono stati riscontrati per ammendanti di origine animale (liquame suino). La mobilità dei metalli pesanti deve quindi dipendere dalla presenza di

metalli in forme solubili negli ammendanti stessi, ma potrebbe anche essere influenzata dall'interazione specifica con il suolo. In generale, il compost è risultato l'alternativa migliore sia dal punto di vista strettamente agronomico (performance produttiva) che ambientale (apporto di metalli e biodisponibilità, stabilità della sostanza organica). Ammendanti stabilizzati come il biochar, che sono più inerti dal punto di vista biologico, nel medio periodo hanno invece esercitato scarsi effetti sulla produttività delle colture in sperimentazione (orzo, fagiolo). Anche gli effetti sul pH (aumento) sono risultati transitori, mentre sembrano più stabili gli effetti sulle proprietà fisiche del terreno (aerazione, densità) e sulla ripartizione dei metalli tra le diverse fasi del suolo. Il biochar infatti, ha favorito la ritenzione di Cu e Zn, mentre potrebbe aumentare la solubilità del Pb, con effetti che possono variare in funzione oltre che della dose anche dell'età del biochar. Infatti, l'ossidazione a carico dei gruppi aromatici del biochar ne modifica le caratteristiche chimiche e quindi le interazioni con i metalli e gli altri componenti del suolo. Anche il biochar comunque, se prodotto a partire da materiali non inquinati, non determina significativi aumenti delle concentrazioni di metalli totali nel suolo e nelle colture, e può quindi essere utilizzato, anche su una scala temporale relativamente ampia, per aumentare lo stock di carbonio dei suoli più che per aumentare la resa produttiva delle colture. I rischi di contaminazione del suolo sembrano scarsi dal momento che gli elementi, che divengono più solubili, sarebbero ridistribuiti verso orizzonti del suolo più profondi e quindi verrebbero diluiti.

Quando invece negli ecosistemi agrari vengono introdotti ammendanti derivati da materiale inquinato, il rischio di contaminazione del suolo e della catena alimentare è concreto. Ammendanti come il biochar o correttivi come la cenere, infatti, a seguito dei processi rispettivamente di pirolisi e incenerimento si arricchiscono di metalli pesanti rispetto al materiale di partenza. In particolare, biochar e cenere prodotti a partire da legno trattato con conservanti a base di rame sono molto ricchi di questo elemento e hanno determinano un forte aumento delle concentrazioni di rame fogliare e nelle radici di girasole. L'aumento di pH conseguente all'aggiunta di biochar e cenere non è quindi in grado di limitare la biodisponibilità e l'accumulo del Cu nella pianta quando questo metallo è presente nell'ammendante in alte concentrazioni. È possibile inoltre che effetti simili siano riscontrabili anche in altre specie e per altri elementi (Cr, As), in caso questi fossero

presenti nel biochar o nella cenere in concentrazioni anomale. La presenza di rame nei tessuti vegetali ha fortemente compromesso la crescita vegetale soprattutto nel caso della cenere derivante dallo stesso legno di partenza, probabilmente perché il Cu era più prontamente solubile, mentre per il biochar la biomassa epigea si è ridotta significativamente (-40%) senza causare moria di plantule. L'utilizzo di biochar e cenere contenenti alte concentrazioni di metalli è quindi da evitare in agricoltura, mentre sarebbe opportuno individuare impieghi alternativi che ne consentano l'utilizzo senza però determinare rischi per l'ambiente o la salute.

Summary

Phytomanagement refers to a group of techniques which use plants to reduce content or toxicity of heavy metals in soils. This thesis focuses on metal phytostabilization, which aims at reducing metal bioavailability in soil. Phytostabilization can occur either in roots or in soils. The first requires the uptake of pollutants and their stable accumulation in root tissues (*in-planta* phytostabilization), the second insolubilization of metals in soil to prevent plant uptake (*ex-planta* phytostabilization). For this thesis both these aspects were explored.

In-planta phytostabilization experiments aimed at evaluating the potential accumulation of heavy metals in rapeseed (*Brassica napus* L. var. *oleifera*) and the time span within metals are retained in degrading taproots before being released into the soil. The effect of increasing sowing density (22, 44, 63 plant m^{-2}) and genotype selection (CHH normal-sized hybrids, semi-dwarf hybrid, and free-impollination variety) on the dynamics of taproot degradation were evaluated (first year) along with the effect of level of soil metal pollution (second year). The results indicated that degradation of root biomass was relatively fast (-83% within 12 months), but after 18 months still 10% of organic matter was available for metal retention. This indicates that the annual supply of root biomass by cultivation can improve metal retention. Metals are mainly retained in the inner cortex, which also owns a higher rate of cellulose and is more recalcitrant to degradation, thus allowing a greater concentration of pollutants to be observable over time in degrading tissues. Nevertheless, after 18 months metal contents was reduced compared with the initial stock, with concentrations depending on the specific metal. The dynamics of root degradation was independent on genotype choice and plant density, but more vigorous cultivars (CHH hybrids) and elevated plant densities should be preferred if the taproots are meant to stabilize metals, because of the higher biomass production (up to 1700 kg ha^{-1} in Taurus at 63 plant m^{-2}). High level of soil pollution (Cd, Co, Cu, Zn) slowed down root degradation due to a reduction in the microbial activity. In addition, the consequent high metal bioavailability was associated to significant increases in root metal contents (and concentrations) despite the degradation process progressed. Overall, despite the degradation of roots cannot be stopped, metal stabilization in taproots is feasible in the long-term and it would be more effective in

polluted soils where it is of paramount importance to reduce metal mobility and accumulation along the food chain.

Ex-planta phytostabilization trials aimed at evaluating the possible risks of soil metal pollution and plant uptake with waste-derived organic amendments. The effects of organic amendments on soil and plants was greatly affected by chemical characteristics of the amendment and its maturation degree. When the amount of organic carbon added to the soil was the same, better productivity and root growth of forage sorghum were obtained with matured compost which is richer in both N and humic substances. None of the tested amendments, i.e., compost from organic urban wastes, anaerobic digestate from plant biomasses, and pig slurry (separated solid fraction) had hazardous contents of heavy metals. Therefore, when the amendments do not derive from polluted feedstock they do not increase the content of heavy metals in the soil or their concentration in plants. However attention should be paid to metal bioavailability, in the middle term some metals (e.g., Ni, Zn) increased significantly increased their mobility, irrespective of the amendment, although generally higher values were found for the animal-derived amendment (pig slurry) which is richer in dissolved organic matter (DOM). Metal mobility in the amended soils therefore may depend on the presence of soluble species in the amendments themselves and probably on the interaction soil-amendment. Compost appeared as the best amendment among those tested for meeting both the agronomic (productivity) and environmental (carbon stock restoration, metal total and bioavailable contents) demands.

Biochar is also an organic stabilized amendment, but it was not found to have relevant effects in the middle term on plant productivity of barley and bean . The effect of biochar on soil properties (pH increases) was also short lived, while the effects on soil physical properties (aeration and bulk density) and metal partitioning in different soil phases appeared longer-lived. Biochar increased Cu and Zn retention, but also the water-soluble Pb, with differences depending on biochar age and application rate. In fact, the oxidation of biochar aromatic rings changes its chemical properties and the interaction with metals. However, when it is produced from unpolluted feedstock, biochar does not increase soil metal contents or plant uptake, probably because soluble metals are distributed to deeper soil horizons,

limiting the accumulation in the rhizosphere. Overall, the real value of biochar lies in the addition of carbon to the soil, rather than in its effect on plants productivity.

On the contrary, when soil amendments are produced from contaminated feedstock, there is a real potential for soil and food-chain contamination. Amendments like biochar and liming agent (e.g. wood ash) concentrate the heavy metals contained in the feedstock material during pyrolysis and incineration respectively. The biochar and wood ash produced from Cu-treated wood in fact were rich in Cu which was available for uptake by plants. The concentration of Cu in sunflower leaves and taproot grown in soil amended with such biochar were greater than those in unpolluted reference soil, while polluted wood-ash severely compromised plant growth (dead of plants) due to the high Cu bioavailability. The increase in soil pH after the addition of amendments was too weak to limit Cu bioavailability when Cu itself was highly concentrated, and this may happen for other metals (e.g., As, Cr) if concentrated in the waste-wood. Above-ground biomass of sunflower was reduced (-40%) in polluted-biochar amended soil, despite plant height was unaffected. Overall, polluted biochar and ash should not be used in agriculture, and alternative uses should be found for polluted wastes.

Introduction: Phytomanagement of heavy metal-polluted soils

Heavy metals (HM) are inorganic elements which naturally occur in soils, water and organisms. HM in soil are component of minerals, and when minerals dissolve due to the weathering, metals are released into the soil solution and can be taken up by plant roots, as well as other elements and nutrients; then, they can either be stored in roots or transferred to edible parts, thus entering the food chain. In the organisms, some metals, like Cu, Zn, Mo, Fe and Ni are considered micronutrients, they being required in small amounts as co-factors of enzymes. Deficiency of these elements has therefore negative impacts on organisms' development and growth (Robinson *et al*. 2009). On the contrary, the presence of non-essential metals (i.e., Cr, Hg, As, Co, Cd) even at very low concentrations is associated to toxicity, since metals cause oxidative stress and inhibition or alteration of enzymes activity and structure, with potentially detrimental effects on cells and organisms (Clemens, 2006).

Since several metals are used for industrial activities, their extraction and release in the environment has increased in the last two centuries. Metals are also released into agricultural soil through fertilizers and pesticides. As a result, many mining and industrial areas and even agricultural lands worldwide suffer from high levels of HM, and the exposure of organisms to toxic concentrations has therefore increased, with negative effects on the environment and human health. Hence, there is need for soil reclamation to avoid further contamination and for reducing the risk of human exposure to toxic elements (Robinson *et al*., 2009).

Many techniques have been developed for soils remediation, and traditionally metal-polluted soils have been excavated and disposed of as special waste or chemically-physically treated to remove the excess of metals (Salt *et al*., 1998). These techniques can effectively remove the pollutants, but they are associated to other drawbacks. Further wastes to dispose of (i.e., polluted sludges after chemical treatments) and non-intended results (e.g., changes in soil properties, possible redistribution of residual metals) should be appropriated addressed (Zerbi and

Marchiol, 2004). In addition, excavation or chemical treatments are not applicable to large areas because of the high cost (Salt *et al*., 1998; Robinson *et al*., 2009).

Beside the traditional treatment methods, phytomanagement, which exploits plants-based techniques for soil reclamation, has emerged in the last decades as a sustainable and environmental-friendly tool to control the fluxes of HM in the plant-soil system. The great interest on green technologies for soil cleaning up is due to the potential for effective *in situ* remediation and the positive side-effects that phytomanagement can exert on the landscape compared to traditional chemical and physical techniques. In fact, phytomanagement appears suitable for cheaply cleaning up extensive areas and limiting soil erosion (Robinson *et al*., 2009; Dary *et al*., 2010). It also has lower impact on water and air quality, improves the landscape and is widely accepted by public opinion (Zerbi and Marchiol, 2004; Mendez and Maier, 2008; Vamerali *et al*., 2010).

Phytomanagement collectively refers to the techniques that manipulate or engineer the plant-soil system and aim at increasing or reducing plant uptake of HM according to the final goal (Robinson *et al*., 2009). Depending on the purpose, phytomanagement can be distinguished in:

- ✓ phytomining;
- ✓ phytovolatilization;
- ✓ biofortification;
- ✓ phytoremediation.

Phytomining is the plant mediated-extraction of valuable metals which are either not economic to mine or present as contaminant in agricultural soils, making them unsuitable for crop cultivation (Nicks and Chamber, 1995). Its feasibility is based on the value of the metal extracted, which depends on the metal itself and the amount extracted (Robinson *et al*., 2009). Phytomining might be feasible for Ni, since some species such as *Berkheya coddii* (Robinson *et al*., 1997) and species belonging to the genus *Alyssum* (Brooks and Robinson, 1998) can accumulate this metal at very high concentrations. In fact, the high biomass productivity of these species allows to extract considerable amount of the metal, up to 144 kg Ni ha^{-1} with *B. coddii* and 121 kg Ni ha^{-1} with *A. bertolonii* (Brooks and Robinson, 1998).

In phytovolatilization, the remediation process involves the volatilization of metals from plant leaves through transpiration. It has been used for the removal of

mercury (Gosh and Singh, 2005) and it might be feasible for Se too (Bañuelos, 2000), which volatile forms are dimethylselenide and dimethyldiselenide (Horne, 2000). It has the disadvantage that there is no control on the chemical species and amount released in the atmosphere (Robinson *et al*., 2009).

Biofortification aims at increasing the concentration of essential micronutrients, like Fe and Zn, in food plants to improve agricultural productivity and human health through reduction of micronutrient deficiency (Branca and Ferrari, 2002). The feasibility of biofortification depends on metal bioavailability. In this regard, the main disadvantage is that the concentration of non-essential metals might also increase along the food chain, especially if cultivation is conducted on contaminated sites (Bañuelos, 2006).

Phytoremediation aims at removing HM from the soil through root uptake and accumulation in the above-ground tissues (phytoextraction) or through metal immobilization in the rhizosphere (phytostabilization). The ability of higher plants to absorb metals and accumulate them in the shoot, sometimes at noteworthy concentrations, is known since the last century, and at present more than 400 species have been recognized to accumulate HM at very high concentrations (Zerbi and Marchiol, *2*004; Robinson *et al*., 2009). Such plants are collectively referred to as hyperaccumulators, due to their exceptional accumulation skills. The thresholds to discriminate hyperaccumulators depend on the normal concentrations range for the metal considered, but conventionally hyperaccumulator species are those that concentrate one metal in the shoots at least 100 times the normal concentration found in plant tissues for that element (Zerbi and Marchiol, 2004). For instance, the threshold for Ni is 1000 mg kg^{-1} (Brooks *et al*., 1998), for Zn is 10000 mg kg^{-1} (Reeves and Brooks, 1983; Reeves *et al*., 1995), whereas for Cd is 100 mg kg^{-1} (Reeves *et al*., 1995).

Due to their accumulation skills, hyperaccumulators have been proposed for the reclamation of metal-polluted soils (Chaney, 1983), and the first field experiments were carried out in the 90s (Baker *et al*., 1994). Despite the high concentrations of HM reached in the shoots, further experiments highlighted that soil remediation using directly such species was difficult because of growth (small biomass) and selectivity (soil, climate, accumulation of only one metal) constraints that limit the extraction effectiveness (Ebbs and Kochian 1997). More recently,

phytoextraction research has focused on fast growing non-hyperaccumualtor species, i.e. *Brassica juncea* (Quartacci *et al*., 2005; Quartacci *et al*., 2006), ryegrass (Zhou *et al*., 2007), *Zea mays* (Luo *et al*., 2005) and fodder radish (Vamerali *et al*., 2011), which combine high biomass production with the ability to absorb several metals, although at lower concentrations than hyperaccumulators. Nevertheless, when high-biomass species are used for phytoextraction, plant growth is mainly limited by metal toxicity (Singh *et al*., 2003). Other constrain factors are the low soil metal bioavailability (Vamerali *et al*., 2010), especially at neutral and alkaline pH, and low translocation from roots to shoots (McGrath *et al*., 2001).

Metals low bioavailability was attempted to be overcome by adding molecules able to foster bioavailability through the formation of soluble complexes, which are easily taken up by plant roots and translocated to the shoot (chemical-assisted phytoextraction). These molecules are called chelators or chelating agents, and can be natural or more often synthetic organic compounds (Luo *et al*., 2005; Quartacci *et al*., 2005; Quartacci *et al*., 2007). Many chelators, i.e. EDTA, NTA and more recently EDDS, have been widely tested in phytoextraction, and despite some positive results, their employment is often associated to some noteworthy disadvantages, such as higher metal leaching (Grěman *et al*., 2001) and phytotoxicity, with reduction in root and shoots biomass (Quartacci *et al*., 2006). In addition, the amount of metals removed from polluted soils through assisted phytoextraction is usually very low compared to the total soil metal content (Quartacci *et al*., 2007), suggesting the several phytoextraction cycles are needed for soil restoration. As an overall judgment, even the assisted phytoextraction has revealed unsatisfactory for metal-remediation purposes (Quartacci *et al*., 2007).

Another technique for soil reclamation is phytostabilization, which has recently emerged as a possible alternative and/or complementary technique to phytoextraction. Phytostabilization aims at maintaining low levels of bioavailable HM, thus limiting the accumulation in the aboveground tissues, and therefore the possible risk of food chain contamination (Vangronsveld *et al*., 1995; McGrath *et al*., 2001). The main objectives of stabilization are (1) to set a vegetation cover and minimize both soil erosion (Mendez and Maier 2008) and surface and groundwater contamination, thanks to elevated transpiration (Gosh and Singh 2005); (2) to limit the uptake of trace elements by crops through the formation of insoluble and not-

bioavailable chemical species (Cunningham *et al*., 1995; Wong, 2003), which reduce the possible contamination of foods (Mendez and Maier 2008) and (3) to reduce the direct exposure of soil organisms to HM and enhance biodiversity (Mendez and Maier 2008). Stabilization of metals can be effectively obtained either by adding amendments such as zeolite or beringite (Mench *et al*., 1999) to polluted soils, or cropping metal-tolerant plants that combine high covering capacities with low metal accumulation in aboveground tissues (low root to shoot translocation).

Combinations of grasses and brushes or trees have been shown to be successful for plant-mediated stabilization, with grasses providing a fast ground cover that temporarily limits wind erosion until shrubs and trees become established (Williams and Currey, 2002). Then, shrubs and trees provide an extensive canopy cover, and their deeper roots prevent erosion over the long term. Furthermore, the presence of different species and habits maintains species and functional diversity, provides a high nutrient environment and improves soil physical characteristics (Belsky *et al*., 1989; Tiedemann and Klemmedson 1973, 2004).

Sometimes, it might happen that growth is severely impaired even for metal-tolerant plants due to high pollution levels. In this case, both amendments for metal immobilization and covering vegetation can be used, since amendments (i.e., beringite) can improve plants' covering capacities, despite very high metal bioavailability; this effect is due to significant reduction of metal translocation to shoots, in both the short and long term (Vangronsveld *et al*., 1995). Other amendments, such as compost, manure and chars, may be used to supply nutrients and increase plants' productivity in both polluted and agricultural soils, but their effects on metal mobility are controversial (Schoenau and Davis, 2006; Hargreaves *et al*., 2008; Sohi *et al*., 2010). Many organic amendments are waste-derived materials, and they contain organic (e.g., PCB, dioxine, PAH) and inorganic (HM) pollutants, thus potentially resulting in a further source of soil contamination (Jones and Healey, 2010).

However, for effective stabilization, plants should be also tolerant to low nutrient and organic matter contents, since many metal-polluted sites are abandoned mining or other industrial areas, where soil fertility and physical structure are severely impaired. A possible strategy to overcome this limiting factor might be the combination of the phytoremediating plants with legume species, since the latter

accelerate microbial activity and improve organic matter content and fertility, thus resulting in a faster restoration (Tordoff *et al*., 2000).

Plant choice is therefore a crucial item for successful stabilization, as highlighted by many studies conducted in polluted areas (Tordoff *et al*., 2000; Freitas *et al*., 2004; Rizzi *et al*., 2004). Many plants able to grow under hard conditions (i.e., high metal bioavailability and poor soil fertility) have been known for long time and belong to selected populations of spontaneous species living in unpolluted soils (Tordoff *et al*., 2000). There is great availability of such plants, since even commercial cultivars of spontaneous species, i.e. *Festuca rubra* L. and *Agrostis capillaris* L., show high metal-tolerance and can be successfully used for stabilizing metal-polluted areas (Vangronsveld *et al*., 1995). However, good candidates for metal stabilization in polluted sites ideally should be native to those areas, as they have evolved survival mechanisms appropriate to the pedo-climatic conditions; in fact, many field trials for phytoremediation resulted in poor plant colonization and soil amelioration since allochthonous species were used instead of native plants of the area to remediate (Mendez and Maier, 2008). Moreover, selecting native plants of the area that must be cleaned up has the advantage to avoid the introduction of not-native and potentially invasive species that may decrease the regional plant diversity.

Much progress has been made in phytotechnologies, but plant-based remediation techniques are still hardly used for land management, because of the long time required for the treatment of polluted soils, the possible competition with crops and the lack of information about the mechanisms regulating metal mobility in soils. In addition, the role of roots and the interaction soil-root-microbes, which has a great effect on metals speciation and mobility in the rhizosphere, is poorly known, probably because the effect is often soil-specific (Robinson *et al*., 2009). This makes it difficult to set generally-accepted guidelines for soil management through phytotechnologies.

In this framework, this thesis explore various aspects of phytotechnologies, with particular regard to the process of *in-planta* phytostabilization and the role of organic amendments. The aims of this study are the acquisition of information on the feasibility of heavy metal stabilization in plant roots and gaining more insight on the effects of soil amendment on content and mobility of metals in soils and in the plant-soil system.

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Chapter 1

Evaluation of the degradation dynamic and potential effectiveness of *in-planta* phytostabilization in rapeseed taproots

Abstract

Phytoextraction and *in-planta* phytostabilization, which aim at accumulating metals in the shoots and roots respectively, might help reduce metal bioavailability and toxicity in polluted soils. Nevertheless, when root are degraded, the metal they had accumulated are released into the soil, and the stabilization is therefore only a temporary solution for metal immobilization. In this study the time span within taproots of *Brassica napus* are degraded in soil and metals released was evaluated in relation to different agronomic condition (cultivar and initial plant density). The results indicate that taproot degradation patterns are different among varieties and densities, but the residual biomass in the long term (18 months) is independent on the genotype and plant density; however, for metal stabilization, the varieties producing larger shoot and root biomass (i.e. Taurus), and higher plant densities (i.e. 44 or 63 plant m^{-2}) should be preferred, since they allow to accumulate higher stock of metals during the cultivation cycle. In taproots, metals are mainly accumulated in the tissues with higher content of cellulose (i.e., inner cortex), which are also more resistant to degradation; therefore, metals more effectively retained in degrading taproots than the biomass. After 18 months there was still 10% of the initial biomass which can retain metals, suggesting that it is possible to increase yearly the organic matter able to accumulate metals through plant litter addition.

1.1. Introduction

Since they grow and develop in the subsoil, roots have been always difficult to study, but nowadays much information is available on roots anatomy, morphology, functions, dynamics of production and death, and the interactions with the soil, due to the development and improvement of root-analyses methods, such as coring (Lauenroth, 2000; Carter *et al*., 2004) minirhizotrons (Vamerali *et al*., 2003), and labeling techniques (Milchunas *et al*., 1985; Milchunas and Lauenroth, 1992).

In addition, at present it is well recognized that roots affect the soil they are in contact with, which is called rhizosphere. Roots in fact improve soil aeration and water absorption, alter the concentration of nutrients and can change heavy metal speciation by releasing chemicals i.e., H^+ and chelants, which acidify the soil and bind heavy metals respectively (Zerbi and Marchiol, 2004), thus increasing the amount of soluble elements in the soil (Robinson *et al*., 2009).

The ability of the root system at interacting with the soil can be exploited to improve the effectiveness of plant-based technologies aimed at reducing metal mobility along the food chain. In fact, vegetation covers the soil and limits the loss of metal through erosion, and some plants may also directly stabilize heavy metals in their roots (*in-planta* phytostabilization). A potentially useful species for *in-planta* phytostabilization is *Typha latifolia* L., which accumulates several metals (i.e. Mn, Co, Cd, Cr, Cu, and As) in roots, most of them being many times (up to 80 times for As, 35 for Cr, 3.5 for Cu and 4.7 for Pb; Varun *et al*., 2012) over the toxicity thresholds reported in the literature. Other species tested for metal stabilization in roots are *Lupinus luteus* L., *Trifolium repens* L., and *Lolium perenne* L. (Dary *et al*., 2010; Lopareva-Pohu *et al*., 2011). *Lupinus luteus* L. can accumulate heavy metals at different extent according to the concentrations in soil, and was reported to retain Cd, Cu, Pb and Zn in roots up to 4.8, 150, 80 and 806 mg kg^{-1} dry weight (dw) respectively in a highly polluted soil. However, this plant was found not suitable for remediation of high metal polluted areas, due to a sharp decrease in both shoot and root biomass as a consequence of metal toxicity, while it is more effective for mildly polluted areas (Dary *et al*., 2010). *T. repens* L., and *L. perenne* L., can accumulate high concentration of metals in roots (139, 204 and 1070 mg kg^{-1} dw in *T. repens* and

110, 470 and 1062 mg kg^{-1} dw in *L. perenne* for Cd, Pb and Zn respectively;) and they are suitable for reducing metals mobility (Lopareva-Pohu *et al*., 2011)

The total amount of metals stored in the root is related to metal concentrations in the roots and root biomass: at equal metal concentration, the more the biomass, the more the amount of metal immobilized. The amount of metals accumulated in roots could be potentially high in crop species, since the remarkable plants' investment for allocation of photo-assimilates to belowground tissues that can lead to high root biomass. In fact, more than 30% of net primary production at global scale is transferred to the soil through root growth (Jackson *et al*., 1997), but the allocation of C into the roots might be higher since values up to 50% of the total C fixed through photosynthesis have been also reported (Nguyen, 2003).

Despite the potential high allocation of carbon and metal accumulation in the roots, when dead roots are broken down the metals previously accumulated are released into the soil. Root death and degradation is a natural process and much research has been carried out to evaluate the life span of roots, especially for fine roots in wood-forest (Guo, 2004; Joslin *et al*., 2006) and shortgrass species (Watson *et al*., 2000; Milchunas *et al*., 2005). The rates of root growth and death have also been measured for some crop species like wheat (Asseng *et al*., 1998) and sorghum (Blum and Arkin, 1984). Nevertheless, there is only little information about root dynamics in crop species such as *Brassicaceae*, that have high potential for metal-accumulation. In addition, to our knowledge, there are no studies considering the relation between root degradation and the efficiency of stabilization of toxic metals in soils over time. As a result, the time span within metals are retained in the roots before being released into the soil is still unknown, and therefore the potential effectiveness of *in-planta* phytostabilization can not be determined.

The aim of this study was therefore to I) evaluate the dynamic of taproot degradation for a model organism (*Brassica napus* L., var. oleifera) in a silty-loam soil and II) metals retention in breaking down taproots to assess the feasibility of *in-planta* phytostabilization, and III) to describe the degradation of taproot in relation to agronomic (cultivar and sowing density) variables. *B. napus* L., was chosen as a model organism in view of its high biomass production and ability at accumulating metals in both shoot and roots, that makes it a potentially suitable species for phytoextraction and stabilization of heavy metals.

1.2. Material and Method

1.2.1. Experimental set up for the cultivation

The experiment was carried out at the experimental farm “Lucio Toniolo” of the University of Padova (Italy). Four cultivars, PR45D01 (semi-dwarf hybrid, Pioneer), Excalibur (CHH hybrid, Dekalb), Viking (open-pollinated variety, NPZ Lembke-Rapool) and Taurus (CHH hybrid, NPZ Lembke-Rapool) of rapeseed were sown on the September 26-27 2008 in the silty-loam soil of the farm in 5.4 × 12 m plots at three different densities (22, 44 and 63 plants m^{-2}), within a three repetitions-split-plot design.

Before sowing, 130 kg ha^{-1} of triple-phosphate and 120 kg ha^{-1} of potassium sulphate, corresponding to 60 kg ha^{-1} of P_2O_5 and 60 kg ha^{-1} of K_2O respectively, were supplied. On the February 17 2009, 80 kg ha^{-1} of nitrogen was supplied as ammonium-sulphate (20.5%).

1.2.2. Set up for the degradation trial

At plants maturity (May 7 2009), 5 plants per plot were collected, thoroughly brushed to remove any particles of soil, and shoot and root fresh and dry (45 °C, 36 h) weight were determined, along with the humidity content. Dry shoots and roots were then milled and digested with HNO_3 for measuring metal concentrations and removals, which were used as reference for the degradation trial.

Nine taproots from each plots were collected on the May 9 2009. After collection, roots were washed as described above, and fresh weight measured. Roots were then stored at 2 °C until they were buried in the in the silty-loam soil of the farm at 15 cm depth (June 3 2009). The roots were wrapped into a 1.2 mm mesh size nylon-net bags (Fig. 1A) to facilitate the following samplings, and encased in a 1.2-cm mesh zinc net (Fig. 1B) to prevent the soil *macrofauna* to enter the net-bags.

A  B 

Figure 1. A rapeseed taproot in a 1.2 mm mesh size nylon-net bag (A) and nylon-net bags in the Zn-mesh net before burying in the soil (B).

The roots were periodically collected and both residual dry biomass and metal contents were measured to estimate the loss of organic matter and metals releasing respectively . At each sampling, the roots were thoroughly washed in plastic boxes filled with deionized water. To avoid loss of root material, the net bags were emptied into 0.2 mm mesh sieves for washing and the the dry weight (105 °C, 24 hours) of taproots was then measured. The degradation experiment run for 18 months, and four sampling were performed over the whole period. Sampling dates for root during the degradation trials are listed in Table 1.

Table 1. Sampling date for the experiment of root degradation.

Sampling Date	DAB*
3 June 2009	0
1 December 2009	181
13 April 2010	314
6 October 2010	490
2 December 2010	547

*DAB = Days After Burying

1.2.3. Analysis of the content of fibers

The content of fibers (Total fiber, ADF; cellulose; lignin, ADL; ash, AIA) in dried (45 °C, 36 hours) taproots was measured at plant maturity according to Van

Soest (1978). The analysis was run for three different tissues: rhizoderm (Rhiz) inner cortex (Cor) and vascular tissue (Cyl) (Fig. 2).

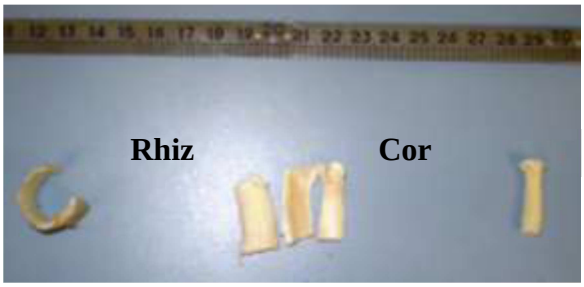


Figure 2. Root tissues: rhizoderm (Rhiz), inner cortex (Cor) and inner cylinder (Cyl).

1.2.4. Heavy metal concentrations in plant

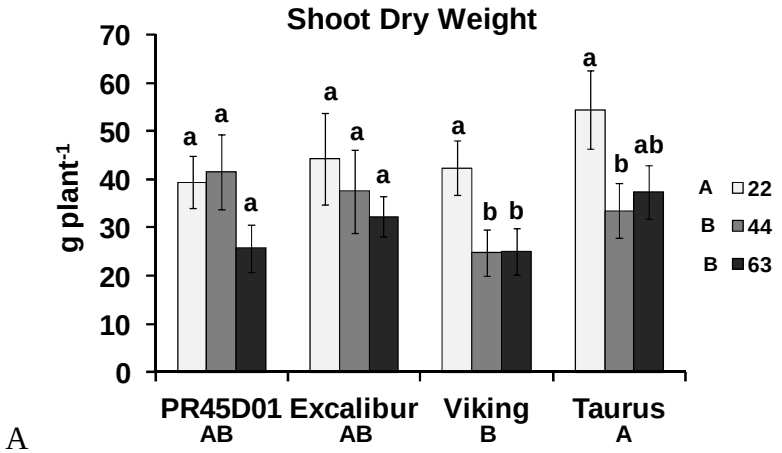
The analysis of the metal concentrations in taproots, taproot tissues (Rhiz, Cor and Cyl) and shoots was performed through ICP-OES (*Inductively Coupled Plasma – Optical Emission Spectroscopy*) after digestion in concentrated HNO_3 according to the USEPA (1995) method.

1.2.5. Statisical Analysis

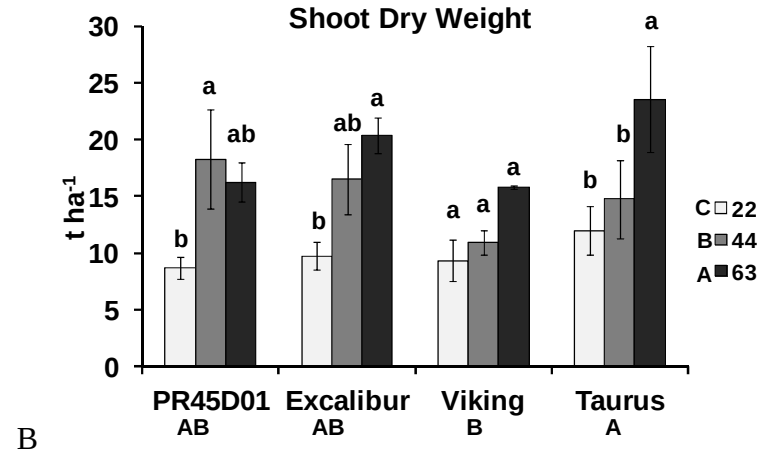
All the analysis were performed in triplicate, except those for the distribution of metals among different root tissues. After checking for normality (Skeweness and Kurtosis tests, $P<0.05$) and homogeneity of variances (Bartlett's test, $P<0.05$), the data were analyzed through ANOVA. (Costat 6.4, Copyright 1998-2008 CoHort Software 798 Lighthouse Ave. PMB 320 Monterey, CA, 93940, USA). The trend of root degradation and metal release were expressed as percentage residual biomass (percentage of the initial dry weight) and percentage residual metal content (percentage of the initial content), respectively, and interpolated over time (number of days after burying) through the Curve-Expert Professional 1.6.3 software (Copyright 2012, Daniel G. Hyams) and running the analysis CurveFinder for the best fit.

1.3. Results

1.3.1. Aboveground and taproot biomass at maturity



A



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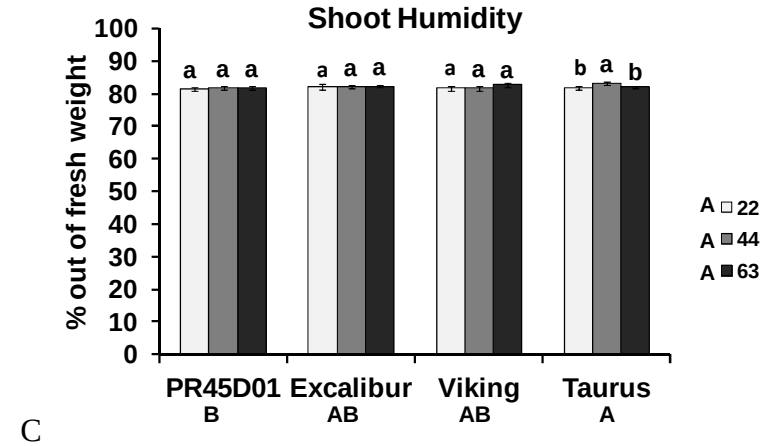


C

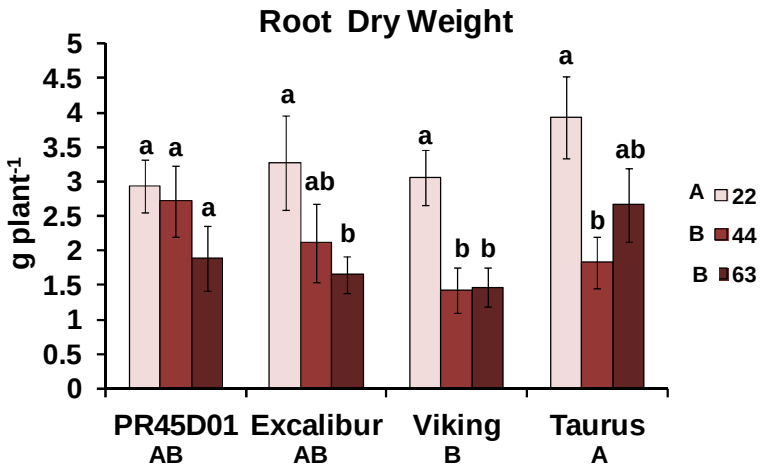
Figure 3. Shoot biomass of four rapeseed varieties at three plant densities, approximately at maturity: dry weight per plant (A) and per hectare (B), and % water content (C). Different letters indicate statistically significant differences (capital letters for main effects; lower case letters among densities within same cultivar) (Test MSD, $P \leq 0.05$). Vertical bars represent standard errors.

Shoot dry weight for individual plants was significantly higher (45 g dw plant^{-1}) at 22 plants m^{-2}, whereas the highest biomass referred to an area (19 t ha^{-1}) was found at the highest density (63 plants m^{-2}) as visible in Fig. 3A and 3B.

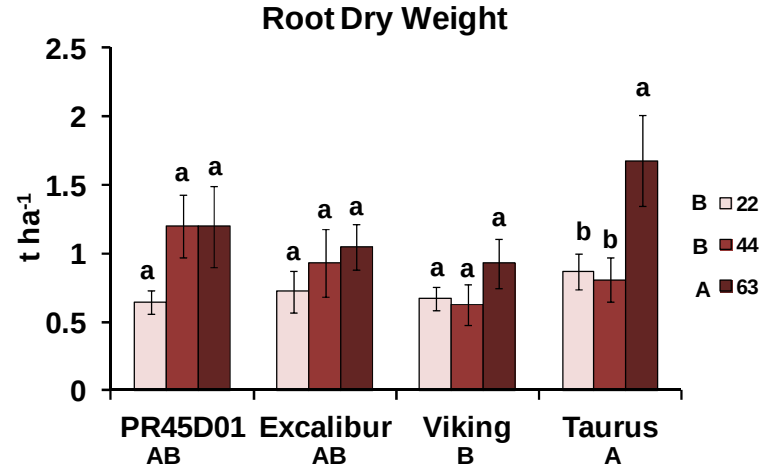
Taurus produced always the highest biomass (41 g dw plant^{-1}), with a statistically significant difference compared to Viking, that produced the lowest biomass (31 g dw plant^{-1}).

The interaction "cultivar × density" was often not significant for individual plant dry weight, but it was generally significant when extending the biomass to an area, with biomass increasing at higher densities (Fig. 3B).

The average humidity content was about 82%, with small differences among the cultivars, whereas no differences were found for the main effect "density" (Fig. 3C).



A



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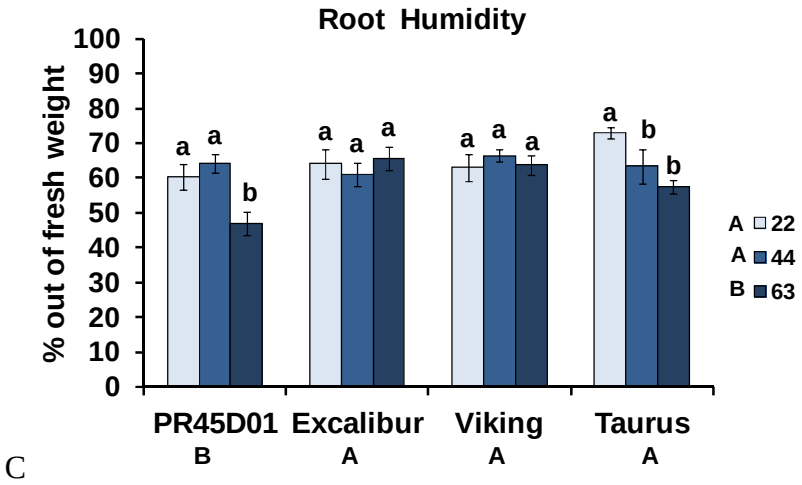


C

Figure 4. Taproot biomass of four rapeseed varieties at three plant densities, approximately at maturity: dry weight per plant (A) and per hectare (B) and % water content (C). Different letters indicate statistically significant differences (capital letters for main effects; lower case letters among densities within same cultivar) (Test MSD, $P \leq 0.05$). Vertical bars represent the standard errors.

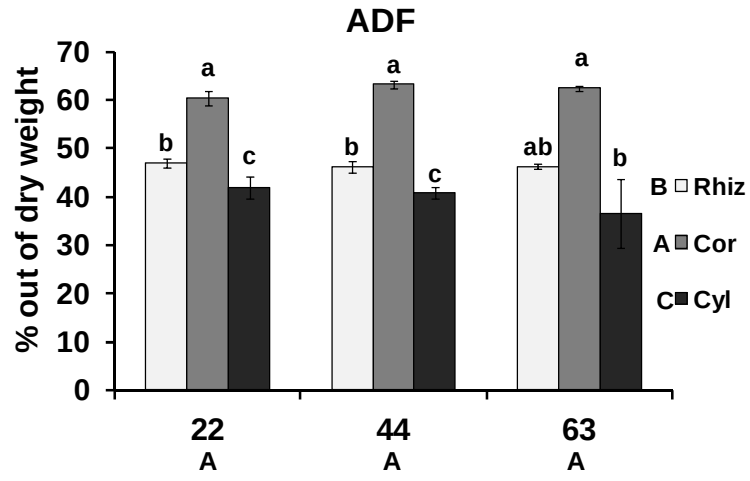
The average dry weight of taproot decreased with increasing density when referred to an individual plant (3.3, 2.1 and 1.9 g plant^{-1} at 22, 44 and 63 plant m^{-2} respectively), but increased when referred to an area (726, 890 and 1211 kg ha^{-1} at the lowest, intermediate and highest density respectively).

Taurus produced the largest biomass (2.81 g dw plant^{-1}, corresponding to 1116 kg dw ha^{-1}) and Viking the lowest (1.98 g dw plant^{-1}, corresponding to 743 kg dw ha^{-1}).

The "interaction cultivar × density" was often significant for both individual and per-hectare biomass, with taproot weight increasing at increasing density.

The humidity content in roots was significantly different among densities, with the lowest value (58.4%) at the highest density. Among the cultivars, Taurus, Excalibur and Viking had the highest humidity content (mean = 64.1%,), with a statistically significant difference (P<0.05) from PR45D01 (57.2%).

1.3.2. Fiber content in taproots



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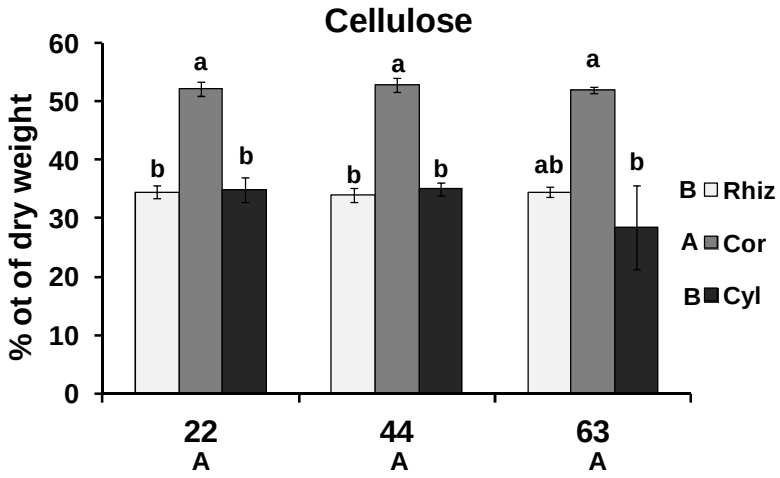

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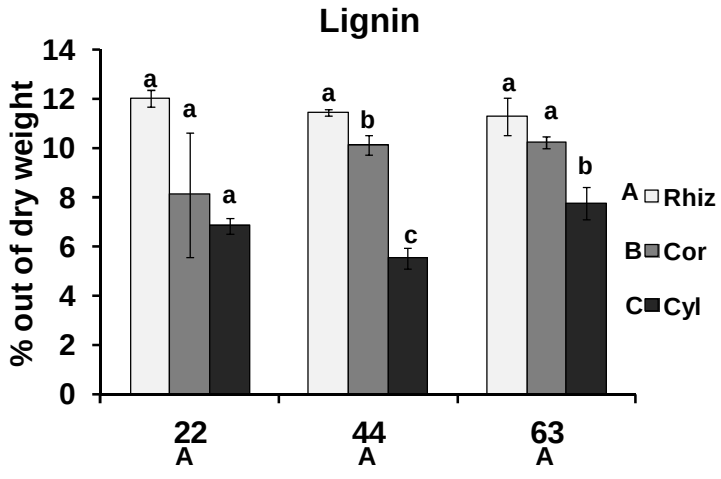

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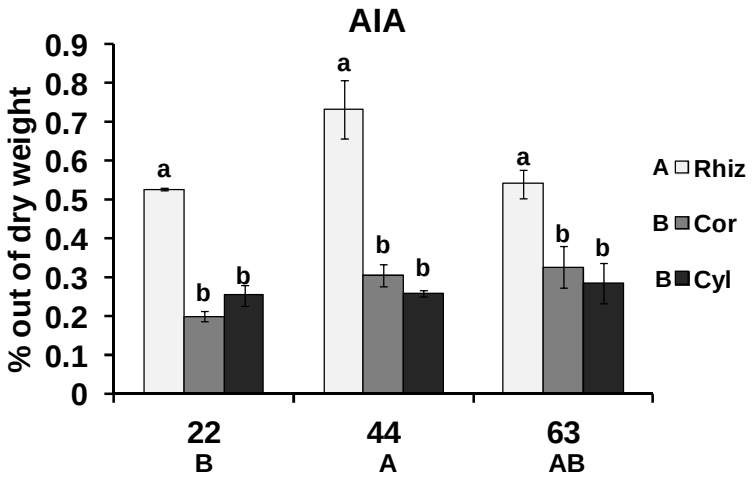


D

Figure 5. Fiber contents (% out of dw) in taproots approximately at plant maturity in different tissues: rhizoderm (Rhiz), inner cortex (Cor) and inner cylinder (Cyl). Different letters indicate statistically significant differences (capital letters for main effects; lower case letters among tissues within same density) (Newman-Keuls test, $P \leq 0.05$). Vertical bars represent standard error.

The content of fibers was analyzed only for the main effect "density" because of the very small amount of material available. Therefore, only the results referring to the main effect "density", "tissue" and their interaction are reported (Fig. 5) and discussed. The average total fiber (ADF), cellulose and lignin content was 49.5%, 39.8% and 9.38% dw respectively, and no significant differences were found for the main effect "density" (Fig. 5A, 5B, 5C). Ash proportion (AIA) was significantly different among densities, with the highest value (0.43% dw) at 44 plant m^{-2}, and the lowest (0.32%) at 22 plant m^{-2} (Fig. 5D).

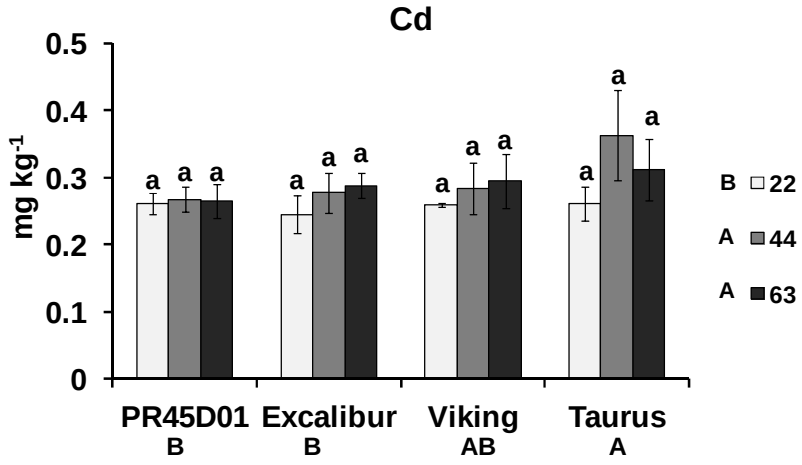
Different tissues had different contents of ADF, lignin and cellulose. The inner cortex (Cor) had the highest ADF (62.1% dw), whereas the inner cylinder (Cyl) had the lowest (39.8% dw) value (Fig. 5A).

The rhizoderm (Rhiz) and Cyl had a lower content of cellulose (mean = 33.5%) than Cor (52.3% dw) (Fig. 5B).

The highest proportion of lignin (11.6% dw) was found in Rhiz, whereas the lowest value (6.71% dw) was found for Cyl (Fig. 5C). Rhiz had the highest content of ash (AIA) (0.7% dw), while the inner Cor and Cyl had a lower value (0.27% dw each) and did not differed from each other (Fig. 5D).

1.3.3. Heavy metals

Metal concentration and removals in shoots



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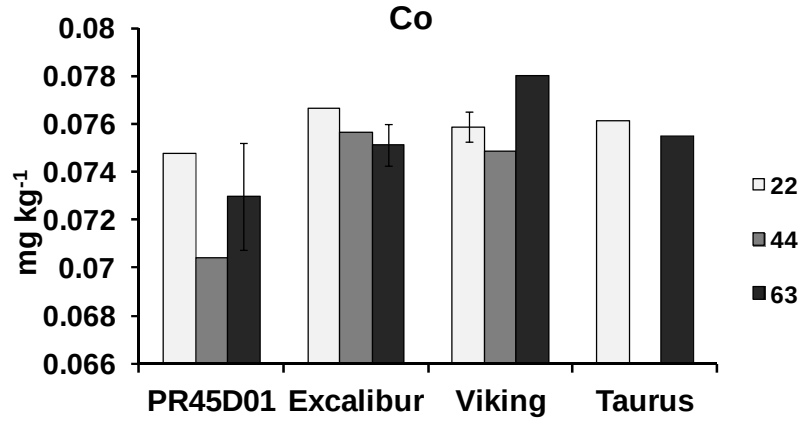

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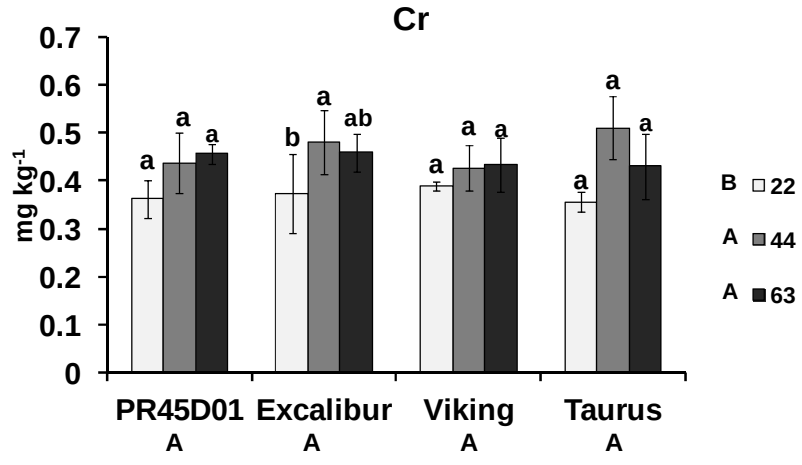

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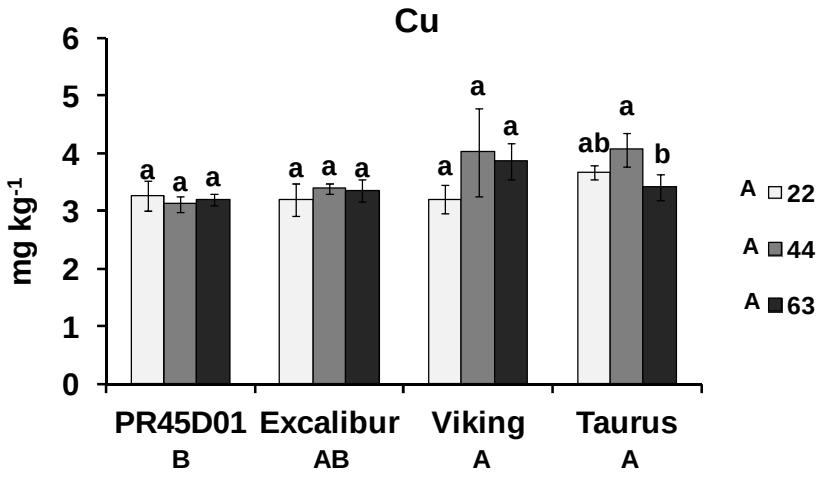

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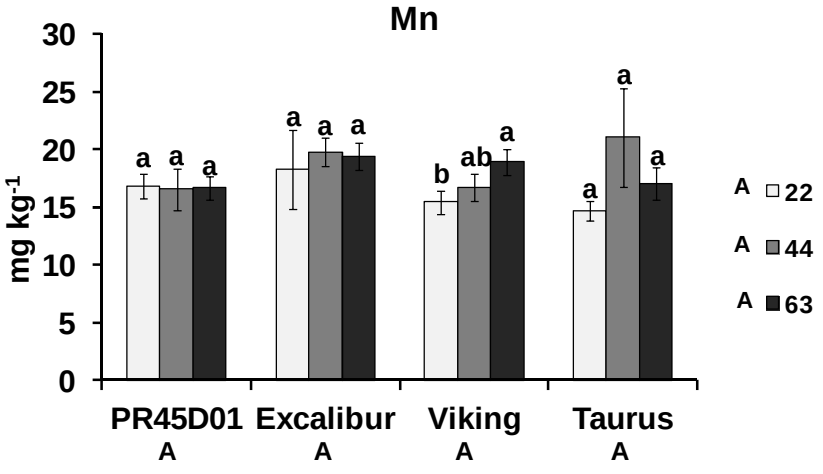

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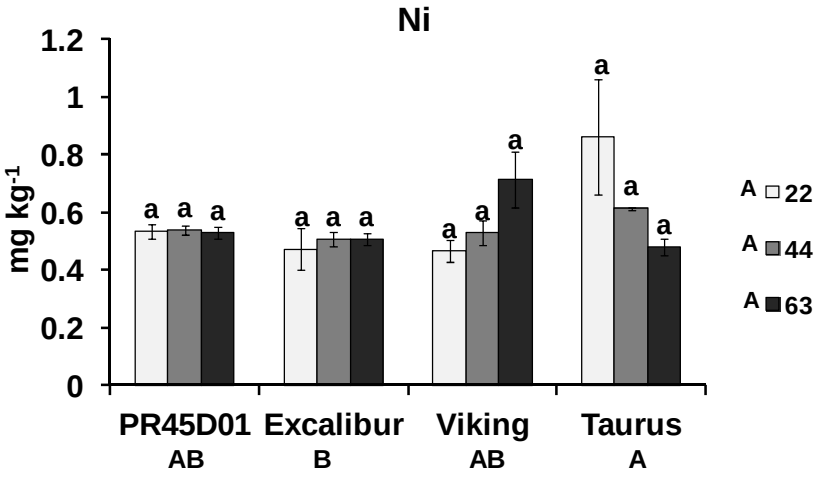

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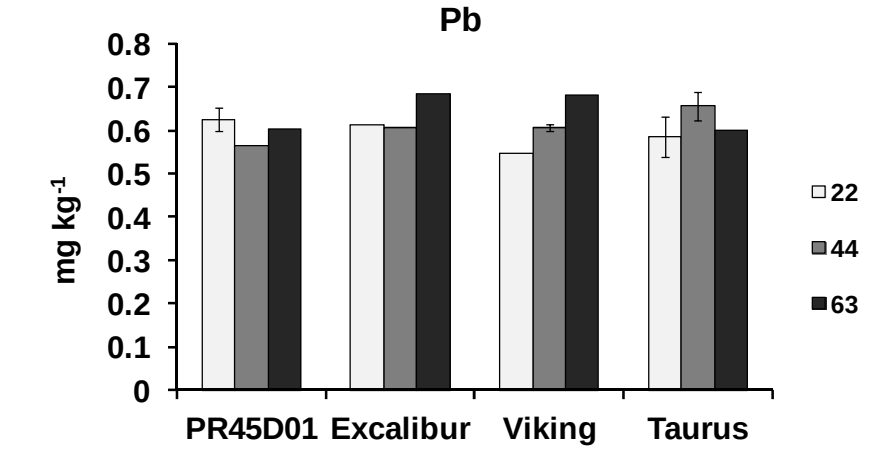

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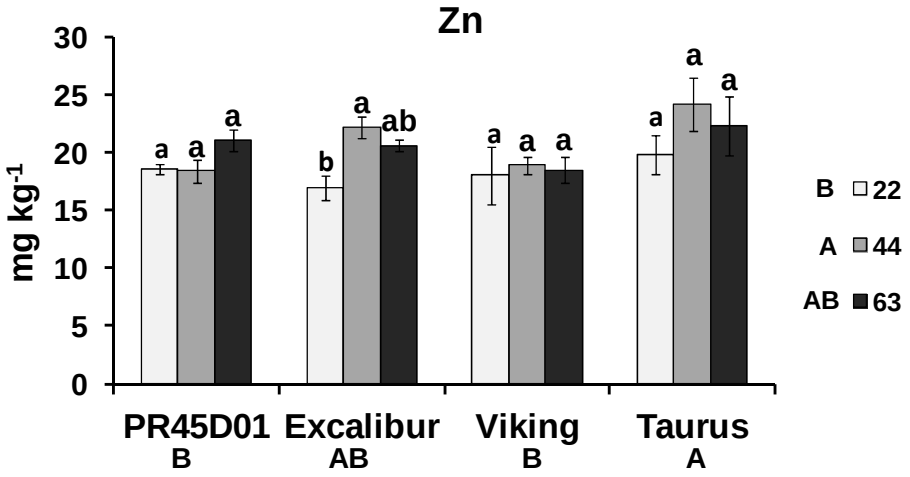

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Figure 6. Metal concentrations (mg kg^{-1} dw) in shoots at harvest of four rapeseed varieties under three plant densities. Different letters indicate statistically significant differences (capital letters for main effects; lower case letters among densities within same cultivar) (MSD test, $P \leq 0.05$). Vertical bars represent standard error.

The concentration of heavy metals in aboveground biomass was in the normal range for all the elements considered. Some metals (i.e. As, Co, Pb,) were not detectable in many samples, therefore, when only few data were available, statistic analysis couldn't be run.

Cd, Cr and Zn reached significantly lower concentration at the lowest density (0.26, 0.37 and 18.37 mg kg^{-1} dw for Cd, Cr and Zn respectively at 22 plant m^{-2}), while no differences were found between the intermediate and highest density. Other elements (Cu, Mn, Ni) were equally concentrated in the three densities (3.48, 17.60 and 0.56 mg kg^{-1} dw for Cu, Mn and Ni respectively).

Among the varieties, statistically significant differences were detected for Cd, Cu, Ni and Zn. Taurus always had the highest concentrations (0.31, 3.71, 0.65 and 22.11 mg kg^{-1} dw for Cd, Cu, Ni and Zn respectively), while PR45D01 had the lowest concentration of Cd (0.26 mg kg^{-1} dw) and Cu (3.19 mg kg^{-1} dw). Viking had the lowest concentration of Zn (18.5 mg kg^{-1} dw), along with PR45D01, and the highest Cu concentration (3.69 mg kg^{-1} dw) along with Taurus. Excalibur had the lowest concentrations of Ni (0.49 mg kg^{-1} dw), and Cd (0.27 mg kg^{-1}dw) along with PR45D01.

The interaction "cultivar × density" was often not significant, but some differences were occasionally found, with higher concentrations at the highest density.

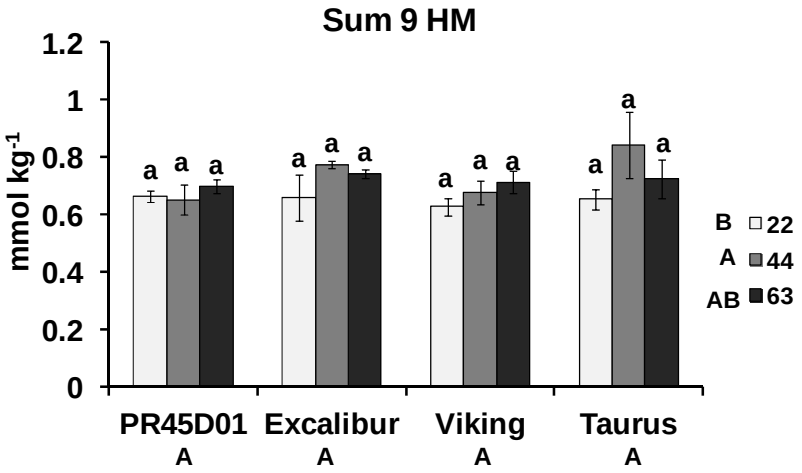
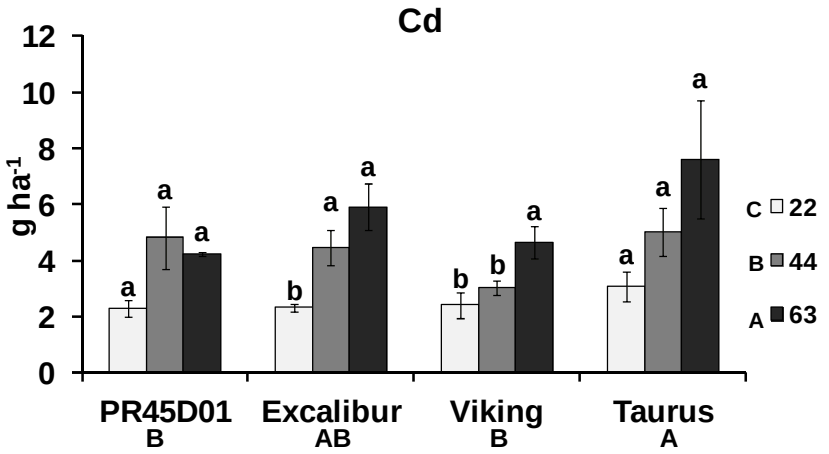


Figure 7. Sum of metal (Cd, Co, Cr, Cu, Mn, Ni, Pb, Zn) concentrations (mmol kg^{-1} dw) in shoots at harvest of four rapeseed varieties under three plant densities. Different letters indicate statistically significant differences (capital letters for main effects; lower case letters among densities within same cultivar) (MSD test, $P \leq 0.05$). Vertical bars represent standard error.

The total concentration of heavy metals did not differ among the varieties, but statistically significant differences were found for the main effect "density", with the intermediate density having the highest (0.74 mmol kg^{-1} dw) concentration and the lowest density (22 plants m^{-2} dw) the lowest concentration (0.65 mmol kg^{-1} dw). The interaction "cultivar × density" was never statistically significant.



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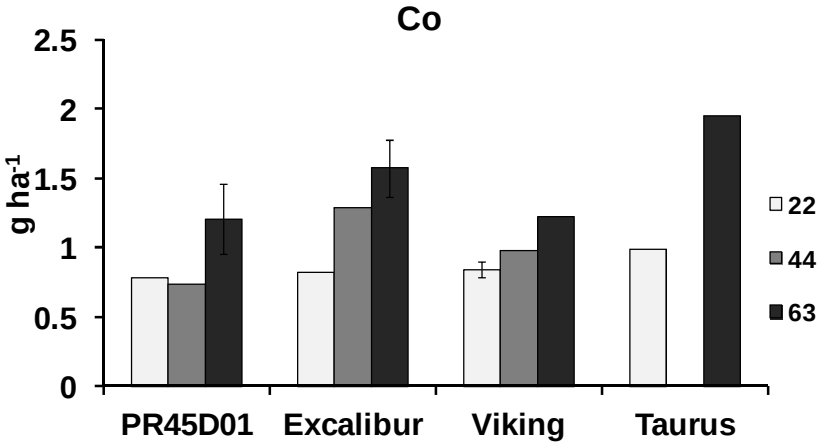

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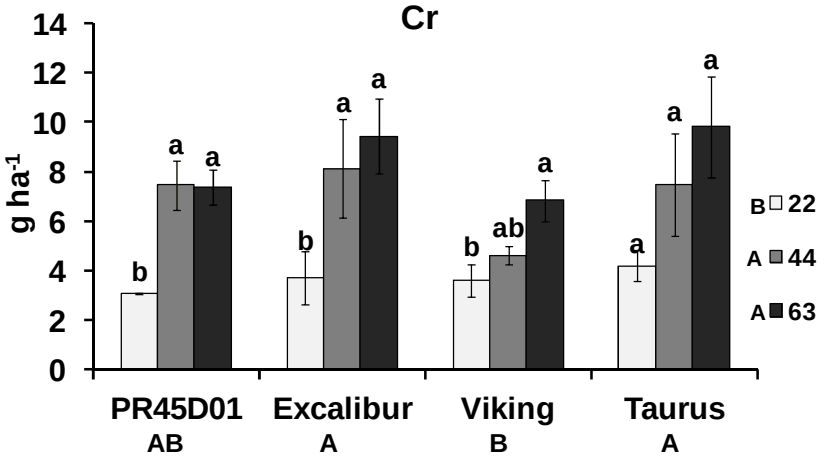

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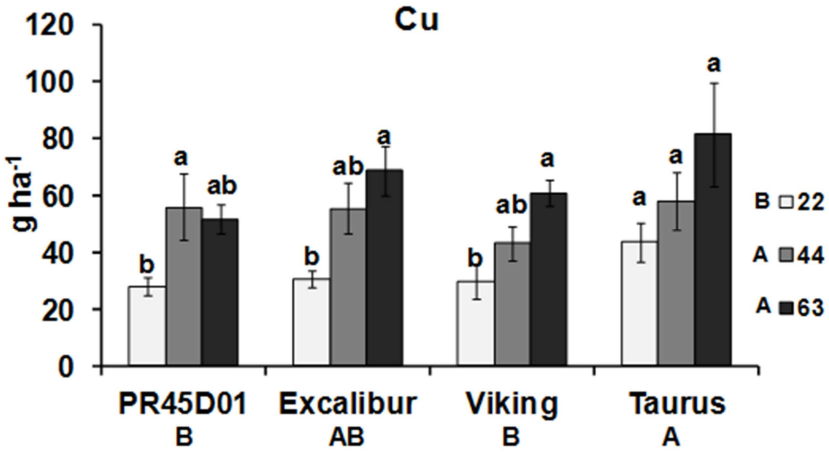

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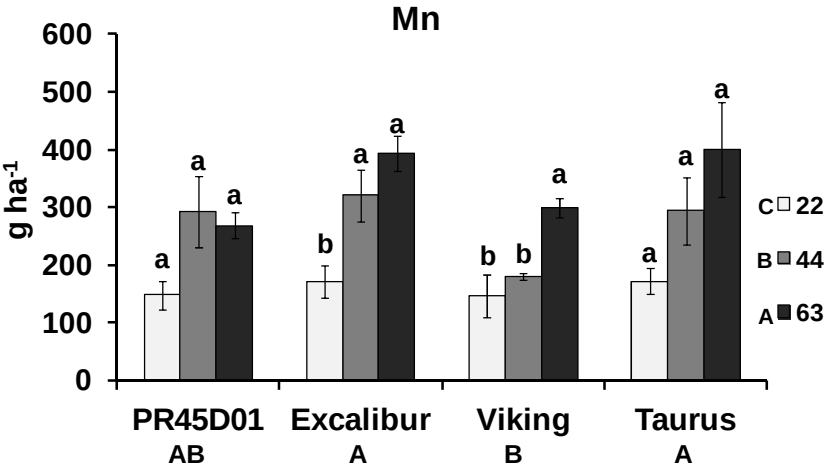

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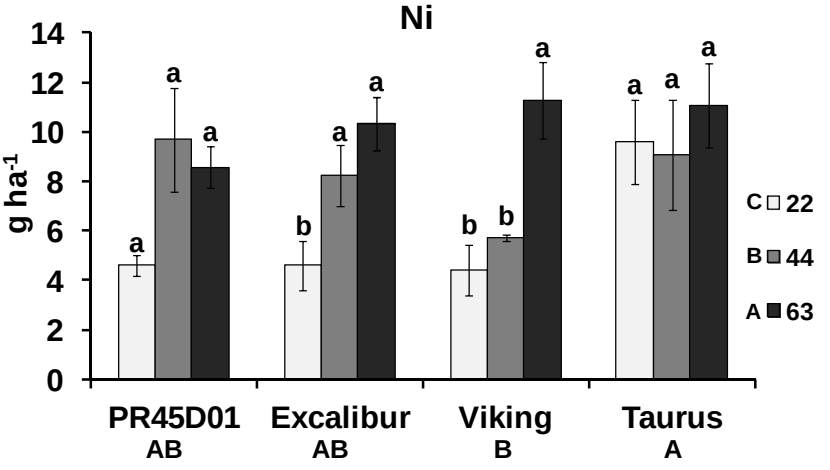

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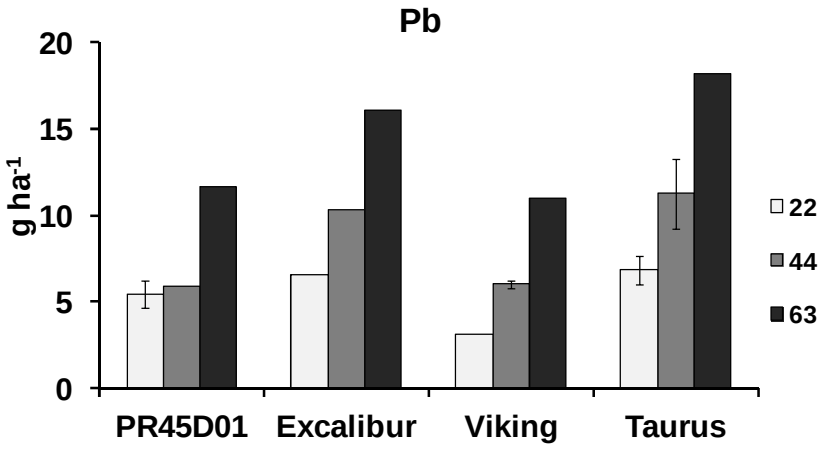

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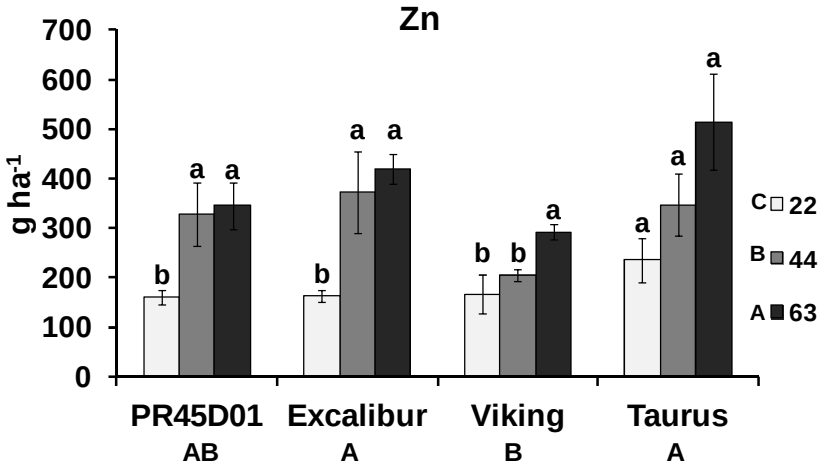

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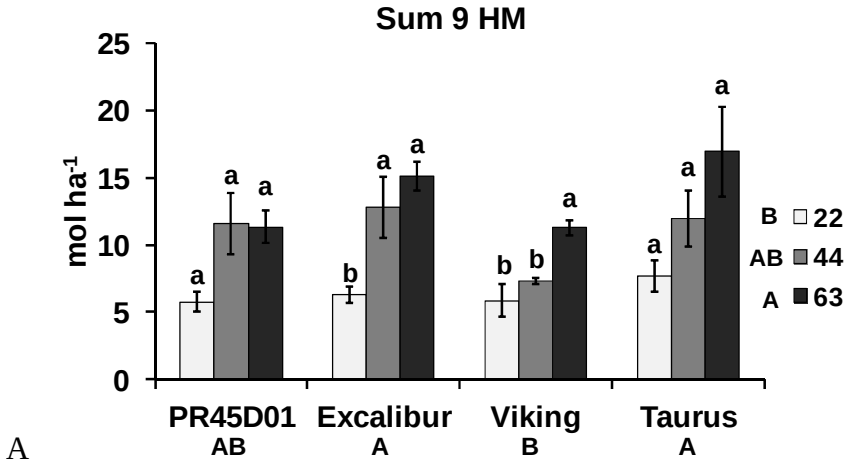
Figure 8. Metal removals (g ha^{-1}) by the above-ground biomass at harvest of four rapeseed varieties at three plant densities. Different letters indicate statistically significant differences (capital letters for main effects; lower case letters among densities within same cultivar) (MSD test, *P*≤0.05). Vertical bars represent standard error.

Metal contents increased with increasing density for all the elements considered.

Among the varieties, Taurus had significantly higher accumulation of metals, followed by Excalibur, whereas PR45D01 and especially Viking were less effective at removing metals by aboveground biomass. However, PR45D01 had relatively high accumulation of Mn, Ni and Zn, with only slight differences from Taurus and Excalibur, thus resulting slightly better than Viking for phytoextraction.

The interaction “cultivar × density” was generally significant, with metal contents increasing with increasing density.



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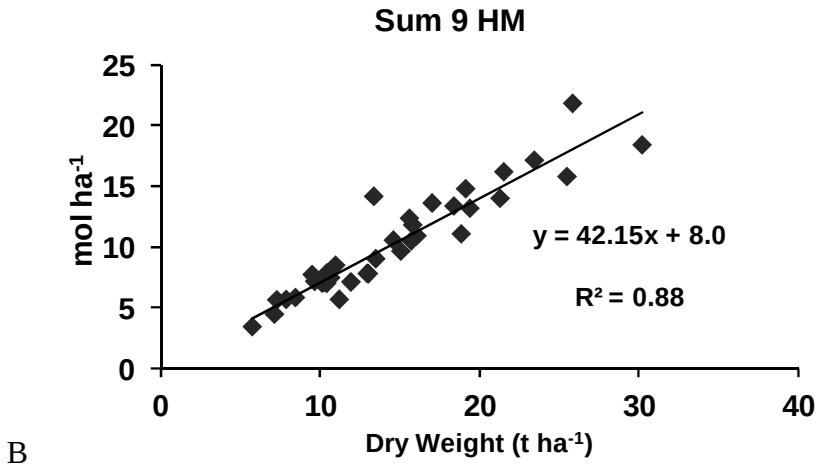


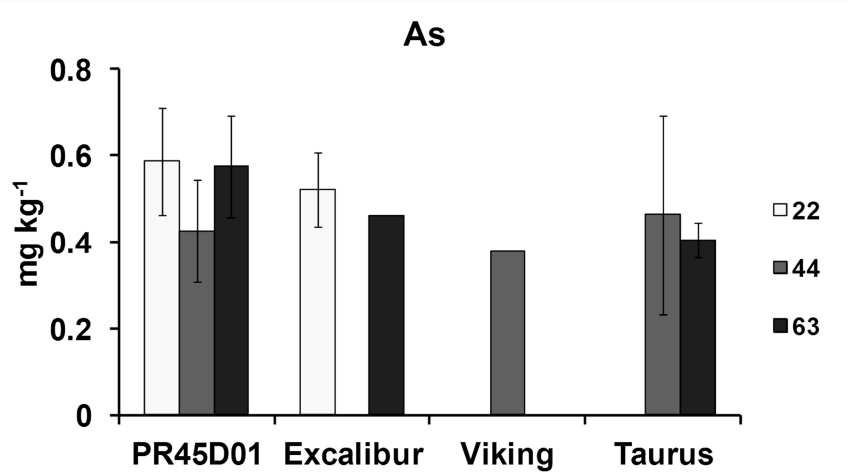
B

Figure 9. Total metal (sum of Cd, Co, Cr, Cu, Mn, Ni, Pb, Zn) removals (mol ha^{-1}) by the above-ground biomass of four rapeseed varieties at three plant densities at harvest. Different letters indicate statistically significant differences (capital letters for main effects; lower case letters among densities within same cultivar) (MSD test $P \leq 0.05$). Vertical bars represent standard error (A); correlation between shoots dry weight (kg ha^{-1}) and total metal removals by shoots (mol ha^{-1}) at harvest (B).

In Fig. 9A is reported the total amount of metals removed through the aboveground biomass. Statistically significant differences were found for both the main effect "density" and "cultivar", with the highest amount of metals (13.7 ± 0.04 mol ha) accumulated at 63 plant m^{-2}, and Taurus and Excalibur being the most effective genotypes at accumulating metals, and Viking the worst.

The interaction "cultivar × density" was significant only for Excalibur and Viking, however all the cultivars showed a tendency at increasing metal removals at 44 and/or 63 plant m^{-2} density. The increasing in metal contents with increasing dry weight followed a linear trend ($R^2 = 0.88$), as showed in Fig.9B.

Metal concentrations and removals in taproots



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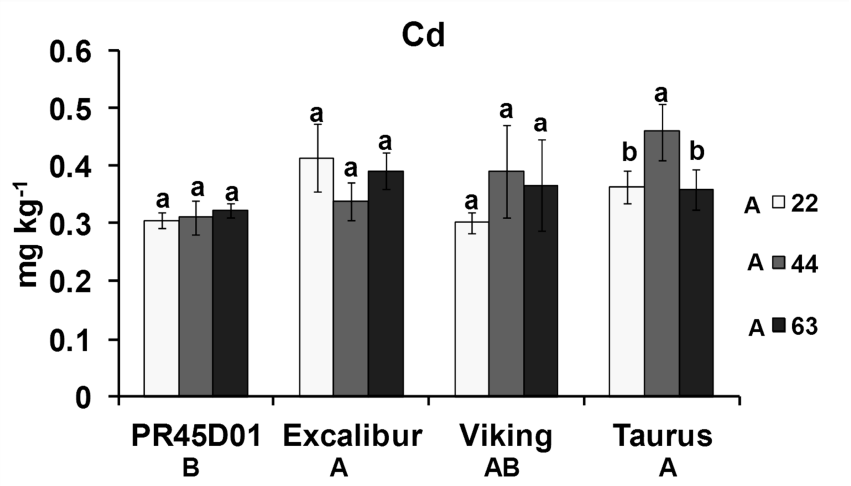

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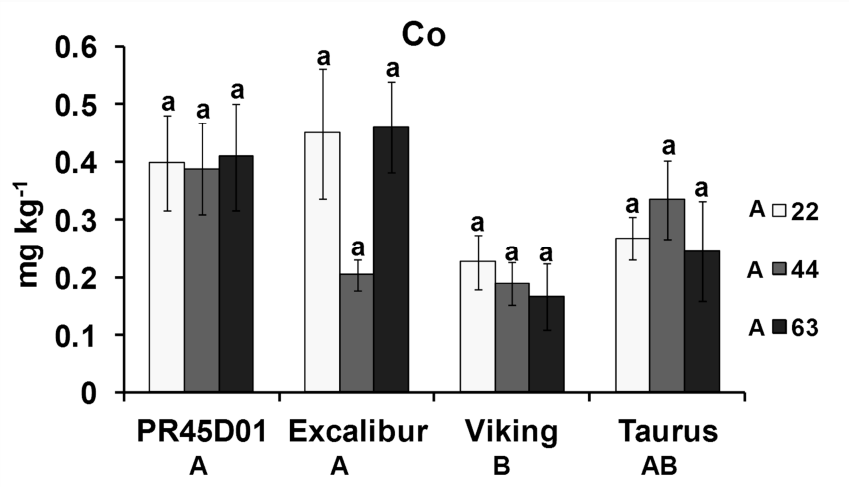

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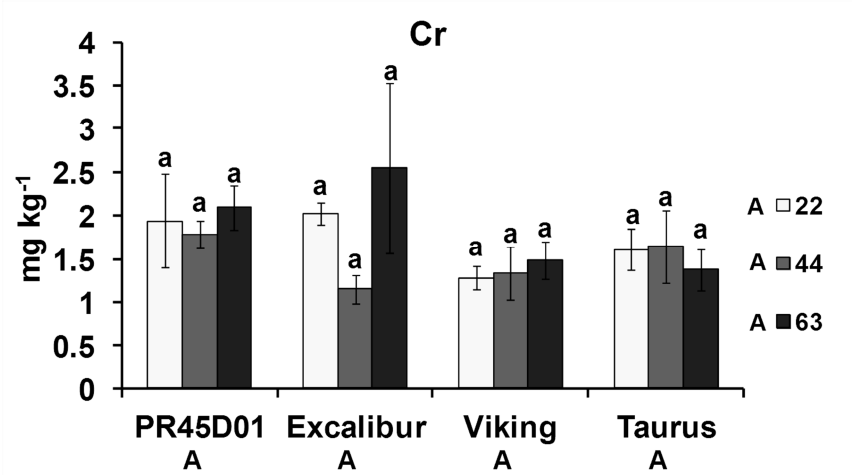

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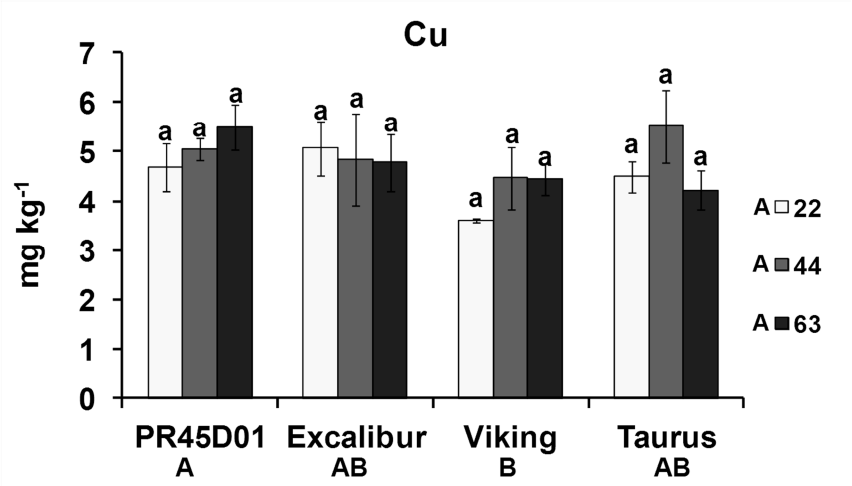

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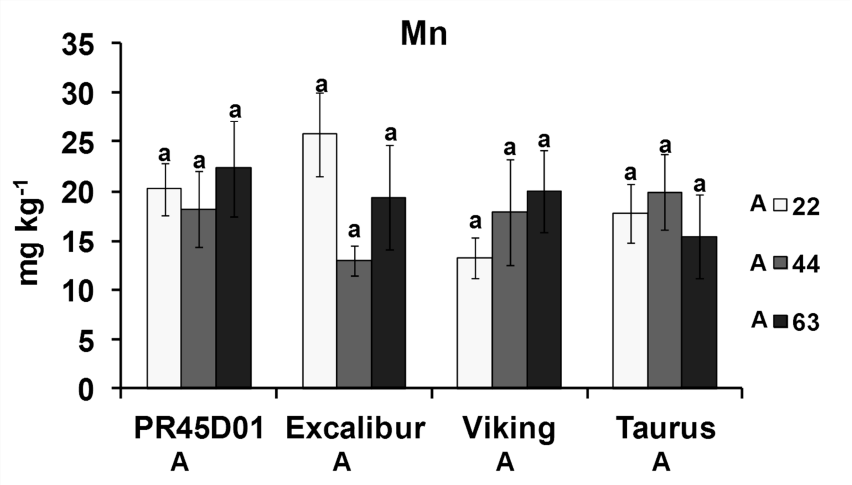

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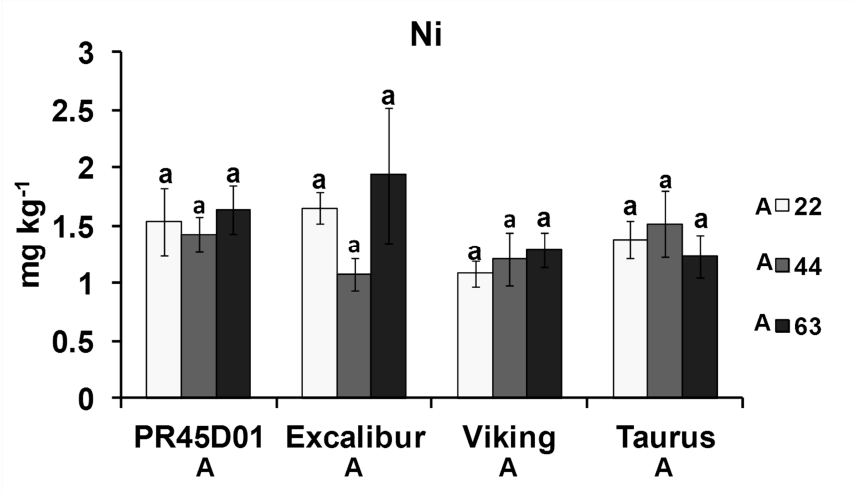

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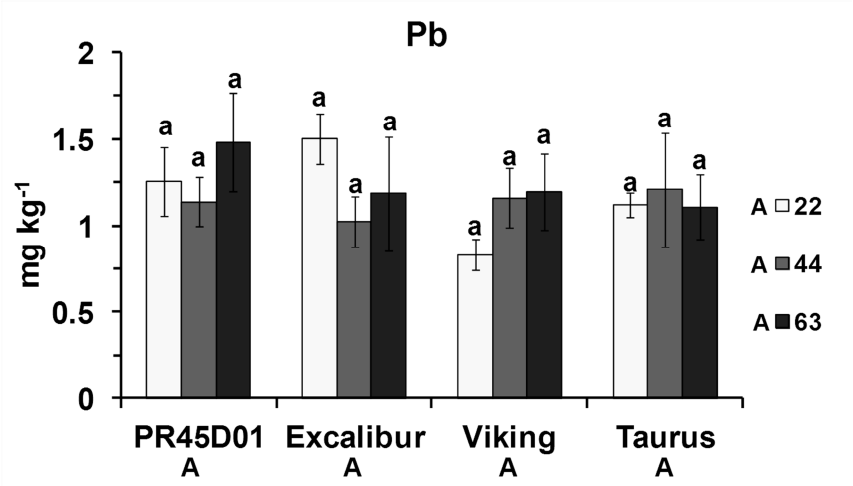

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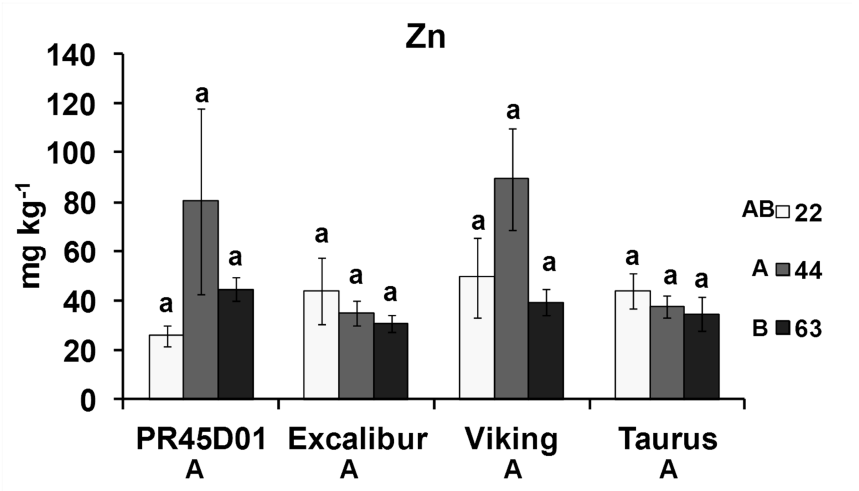


I

Figure 10. Metal concentrations (mg kg^{-1}) in taproots of four rapeseed varieties at three plant densities at harvest. Different letters indicate statistically significant differences (capital letters for main effects; lower case letters among densities within same cultivar) (MSD test, $P \leq 0.05$). Vertical bars represent standard error.

Metal concentrations in roots did not differed significantly among the densities, except for Zn, that reached the highest concentration (60 mg kg^{-1} dw) at 44 plant m^{-2} (Fig. 10I).

Different cultivars had significantly different accumulation of Cd, Co and Cu. PR45D01 accumulated the highest concentrations of Co (0.40 mg kg^{-1} dw) and Cu (5.07 mg kg^{-1} dw), and the lowest of Cd (0.31 mg kg^{-1} dw). Viking accumulated the lowest concentration of Co (0.19 mg kg^{-1} dw) and Cu (4.17 mg kg^{-1} dw), wheres Taurus and Excalibur accumulated the highest concentrations of Cd (mean = 0.39 mg kg^{-1} dw). Excalibur had the highest concentration of Co (0.37 mg kg^{-1} dw) along with PR45D01.

The interaction "cultivar × density" was never significant, except for Cd in Taurus, where the highest concentration was found at the intermediate (44 plant m^{-2}) density (Fig. 10B).

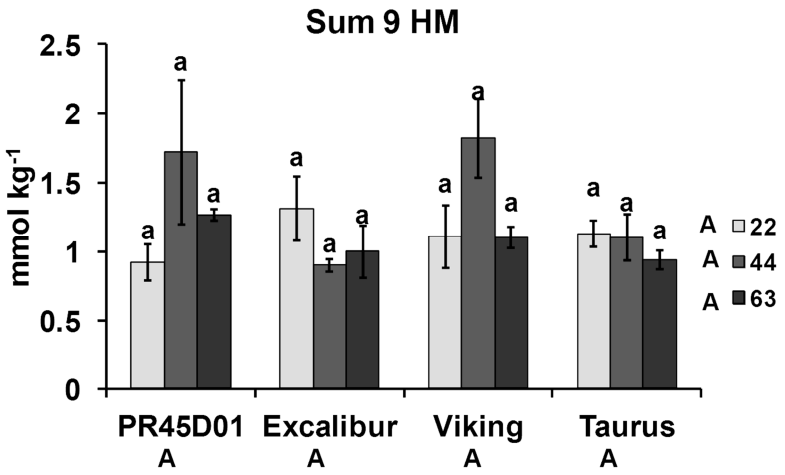
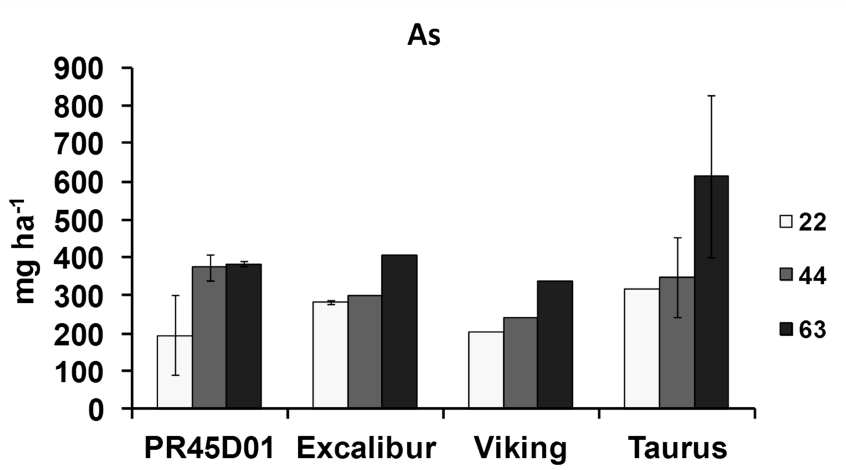


Figure 11. Total metal (sum of Cd, Co, Cr, Cu, Mn, Ni, Pb, Zn) concentrations (mmol kg^{-1} dw) in roots at harvest of four rapeseed varieties under three plant densities. Different letters indicate statistically significant differences (capital letters for main effects; lower case letters among densities within same cultivar) (MDS test, $P \leq 0.05$). Vertical bars represent standard error.

The overall concentration of metals in taproots (sum of nine elements; Fig. 11) was not significantly different among "density" or "cultivar" or their interaction (mean = 1.2 mmol kg^{-1}).



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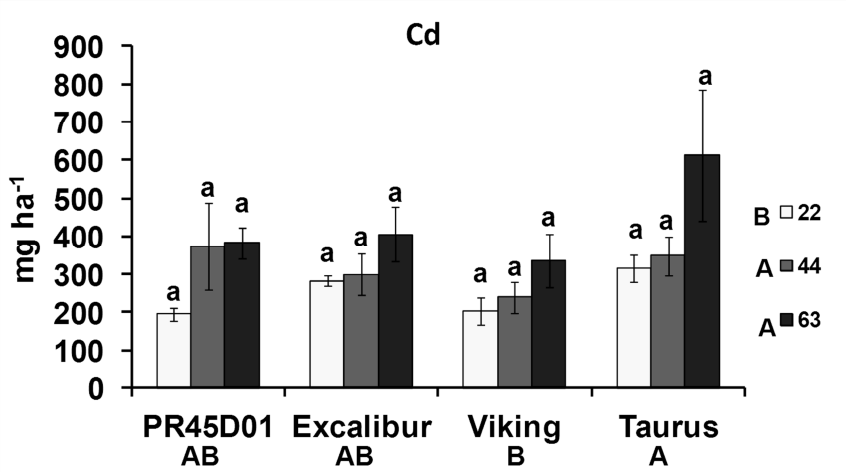

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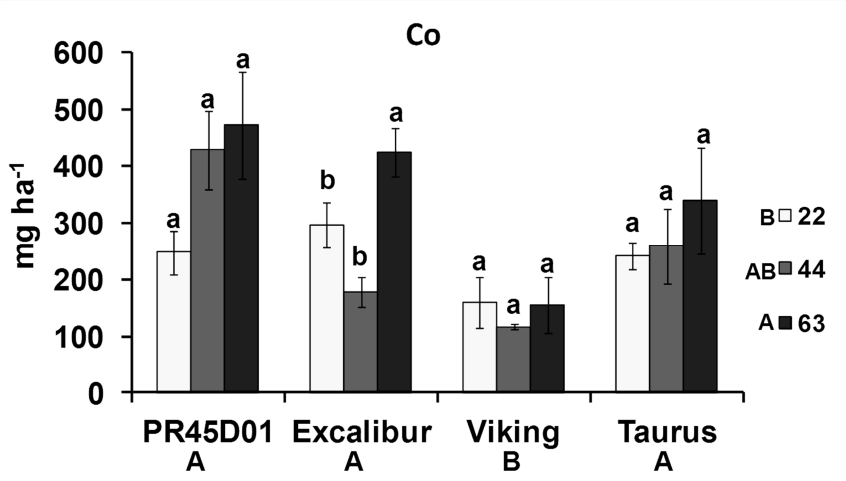

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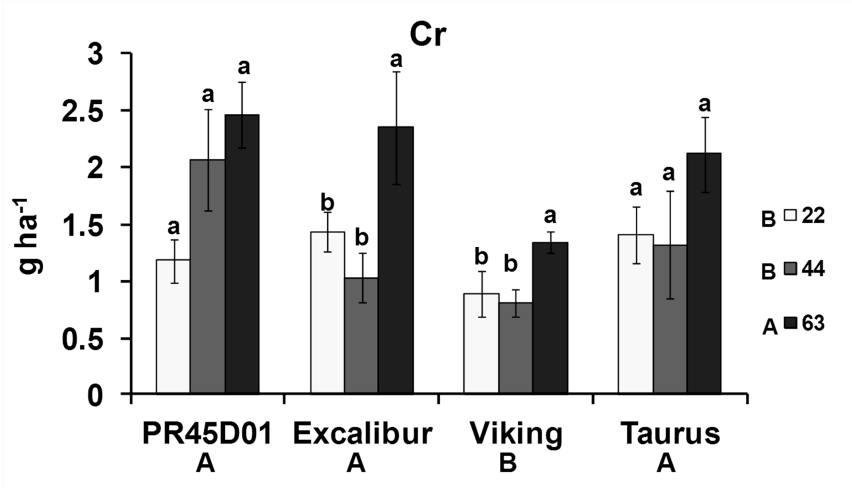

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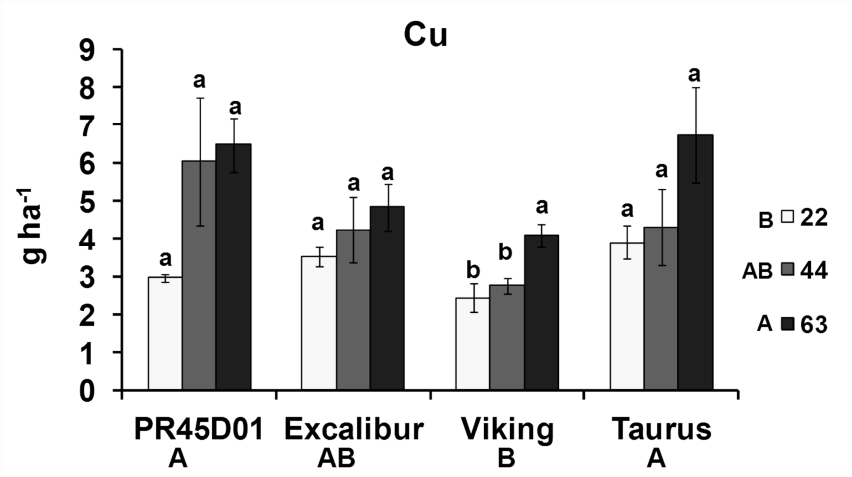

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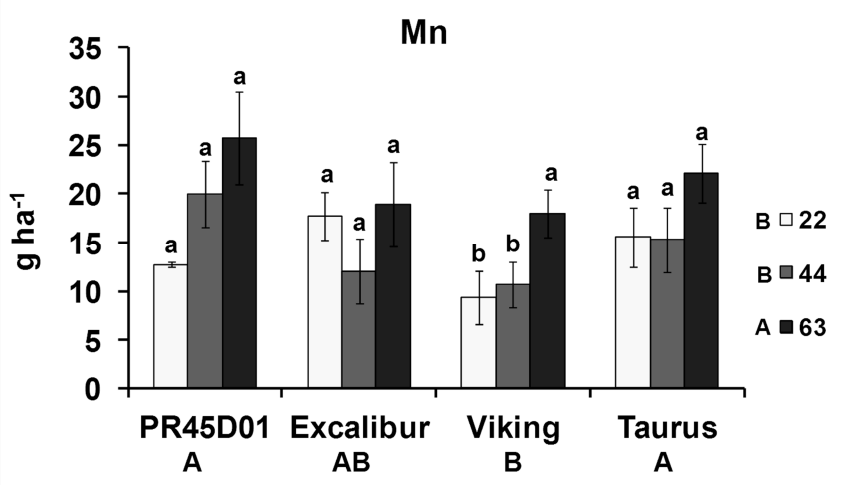

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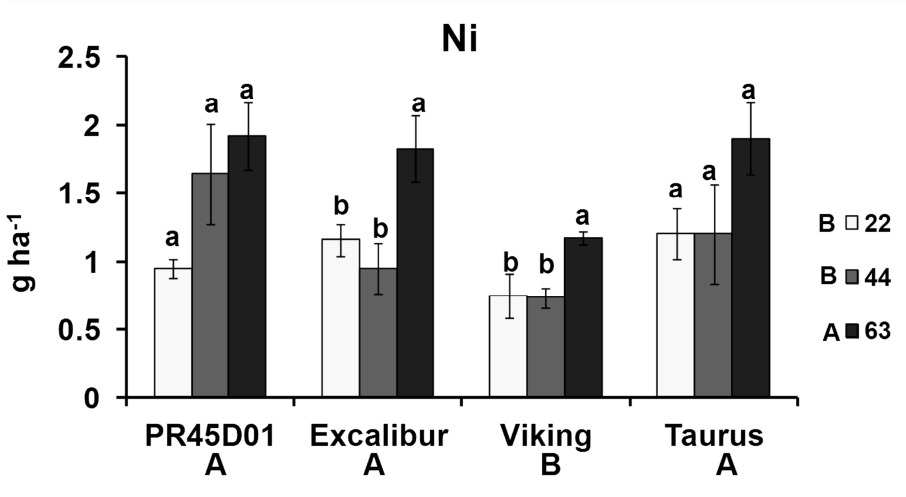

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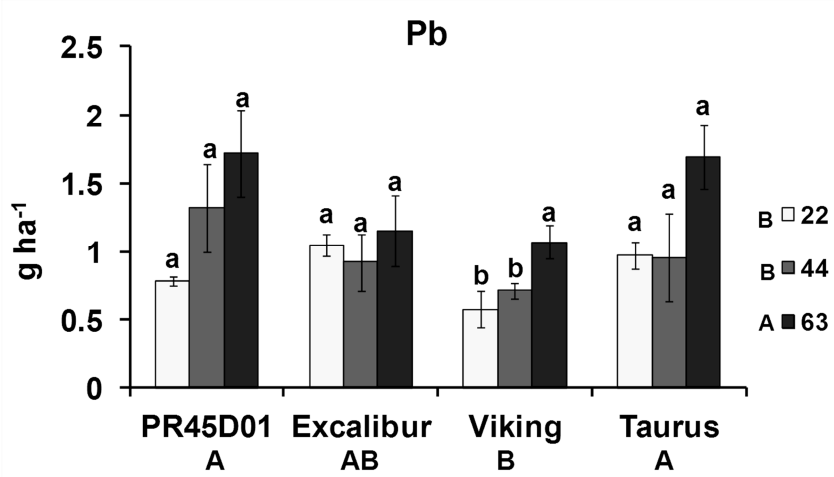

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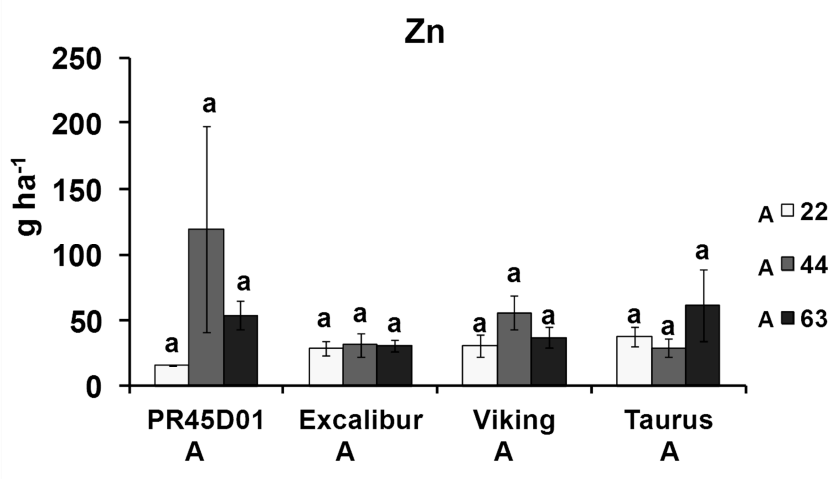

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Figure 12. Metal removals by the taproots biomass of four rapeseed varieties at three plant densities at harvest. Different letters indicate statistically significant differences (capital letters for main effects; lower case letters among densities within same cultivar) (MSD test, $P \leq 0.05$). Vertical bars represent standard error. All values are expressed in g ha^{-1}, except As, Cd and Co which are expressed in mg ha^{-1}.

The accumulation of metals in rapeseed taproots increased with increasing density, with statistically significant differences from the lowest to the highest density.

PR45D01 and Taurus had the highest metal contents, with generally significant differences from Excalibur and Viking. However, Excalibur performed better than Viking for Co, Cr, Ni and slightly better for Cd, Cu, Mn, Pb.

The interaction "cultivar × density" was significant for some metals, but only for Excalibur and Viking. Co, Cr and Ni contents in taproot significantly increased with increasing density for Excalibur and those of Cr, Cu, Mn and Ni increased with density for Viking.

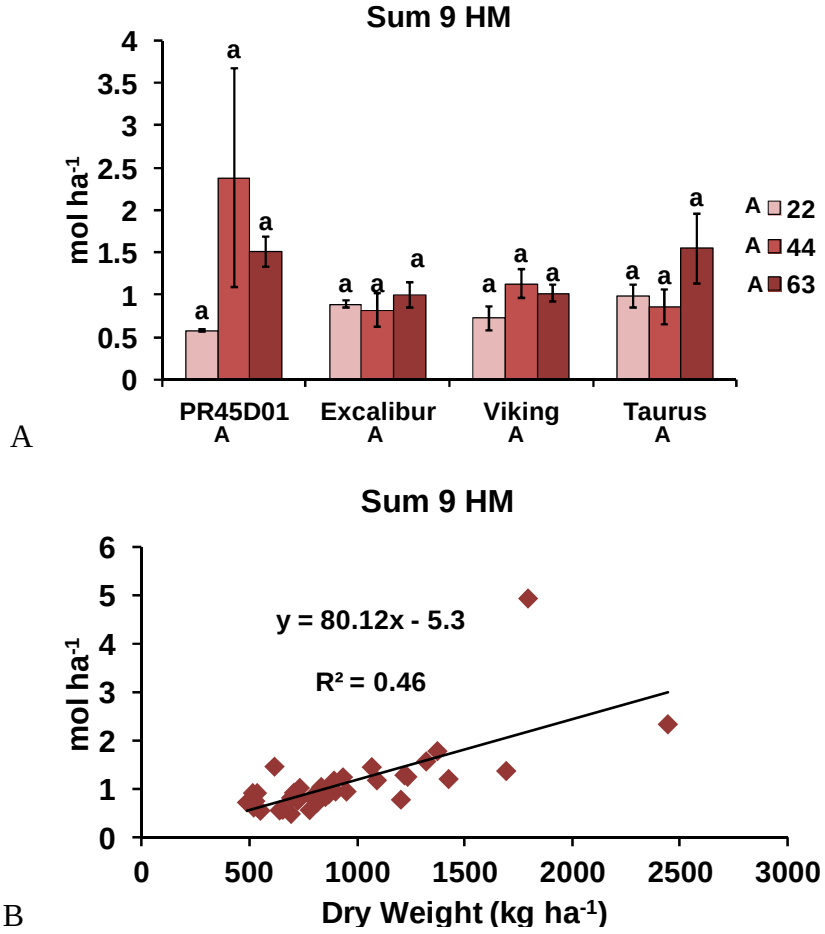
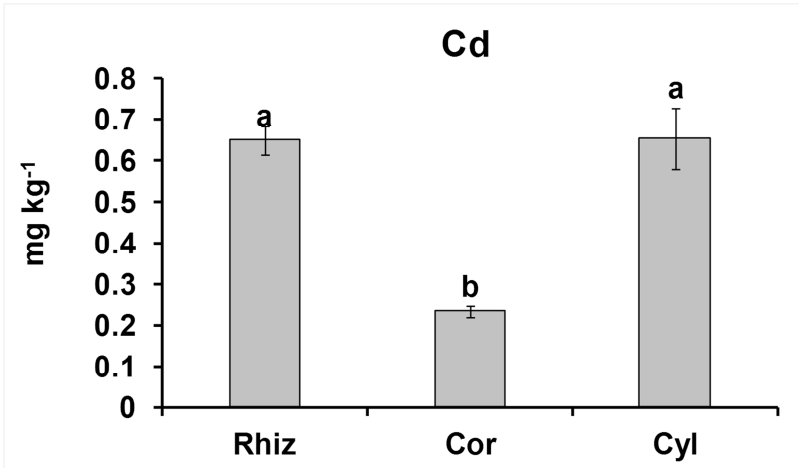


Figure 13. Total metal (sum of As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Zn) removals (mol ha^{-1}) by four rapeseed varieties at three plant densities at harvest. Different letters indicate statistically significant differences (capital letters for main effects; lower case letters among densities within same cultivar) (MSD test *P*≤0.05). Vertical bars represent standard error (A); correlation between root dry weight (kg ha^{-1}) and total metal removals by roots at harvest (B).

When considering the total amount of metal recovered in roots no significant differences were found either for density, cultivar or their interaction (mean = 1.1 mol ha^{-1}; Fig. 13A). The correlation between the taproot biomass and the total metal removals is weak as visible from the low R^2 (Fig. 13B).

1.3.4. Metal concentration and distribution in root tissues



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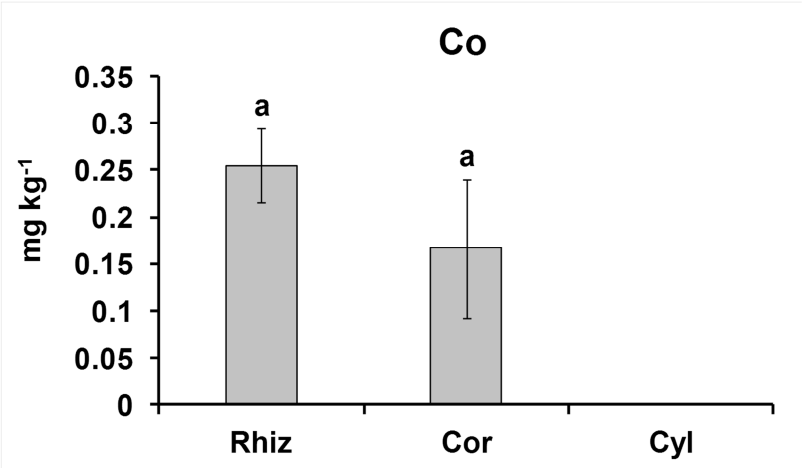

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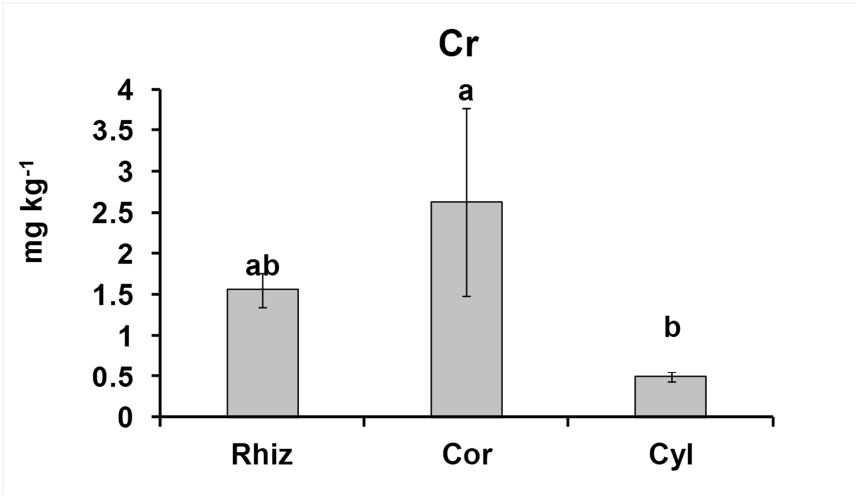

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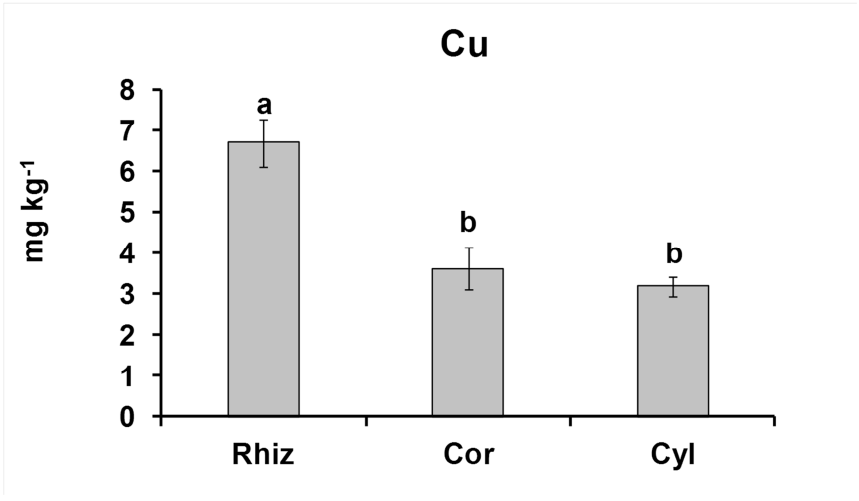

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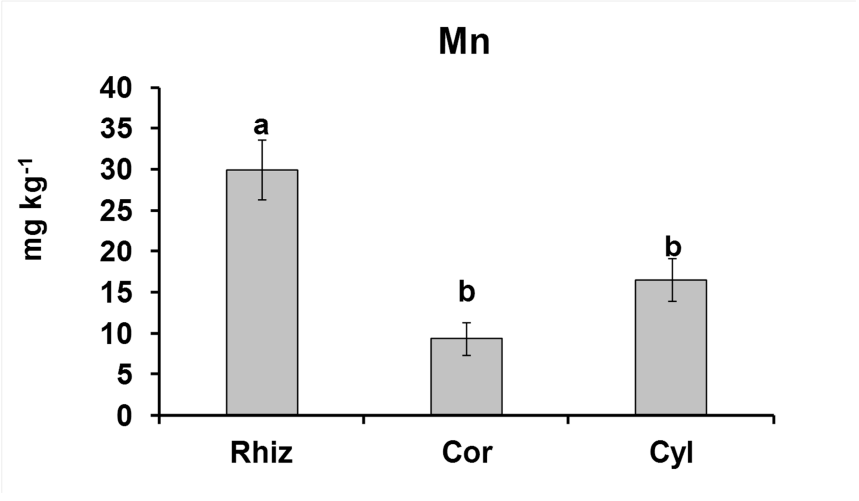

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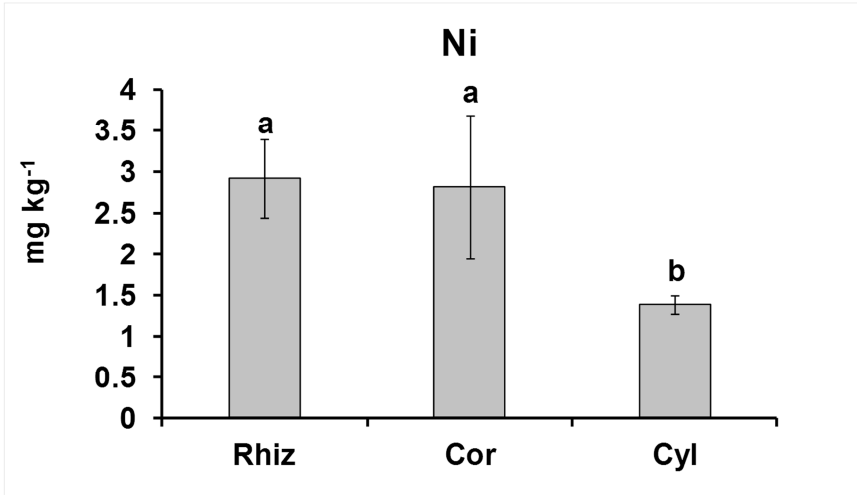

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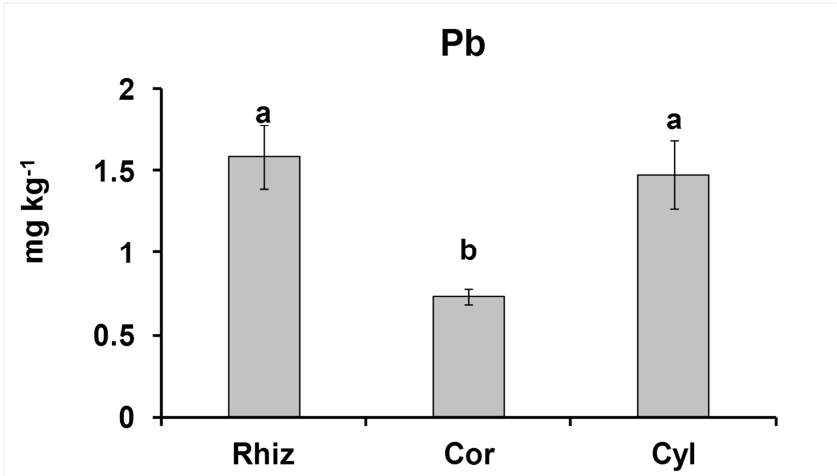

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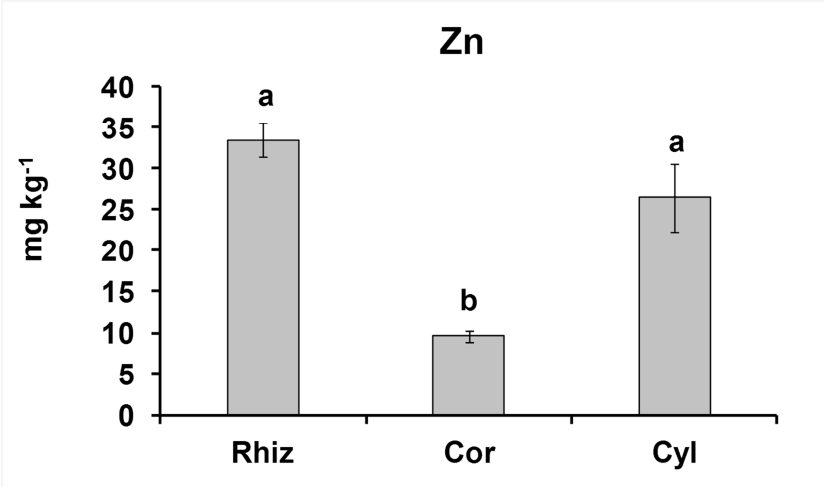

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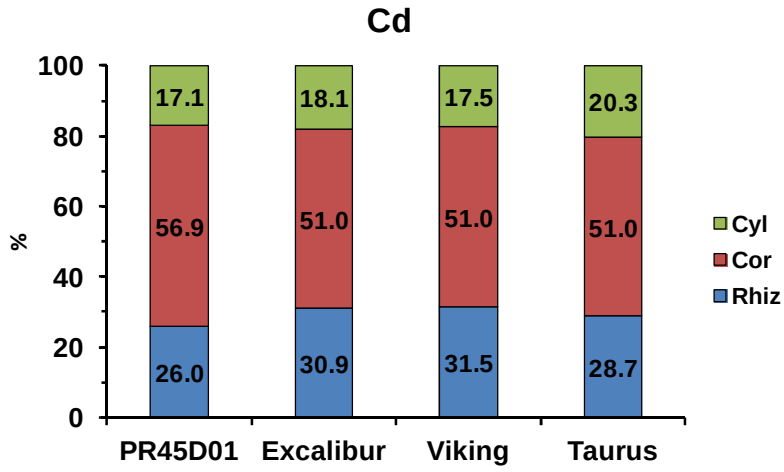
Figure 14. Metal concentrations (mg kg^{-1} dw) in taproot tissues: rhizoderm (Rhiz); inner cortex (Cor); inner cylinder (Cyl). Different letters indicate statistically significant differences (LSD test, $P \leq 0.05$). Vertical bars represent standard error.

Heavy metals had different concentrations depending on the root tissue (Fig. 14). The highest metal concentrations were generally found in Rhiz, whereas the lowest in Cor. Exceptions are Co, Cr and Ni: Co and Ni had the same concentration in the Rhiz and Cor (mean = 0.21 mg Co kg^{-1} dw and 2.86 mg Ni kg^{-1} dw; Fig 14B and 14F); Cr was more concentrated (2.63 mg kg^{-1} dw) in Cor than in the other tissues (Fig 14C). Cu and Mn were equally concentrated in Cor and Cyl (3.4 mg kg^{-1} dw and 12.9 mg kg^{-1} for Cu and Mn, respectively; Fig. 14D and 14E).



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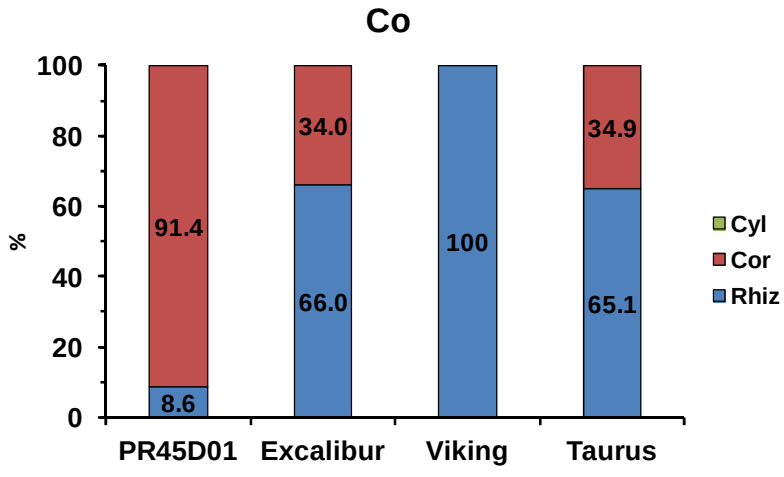

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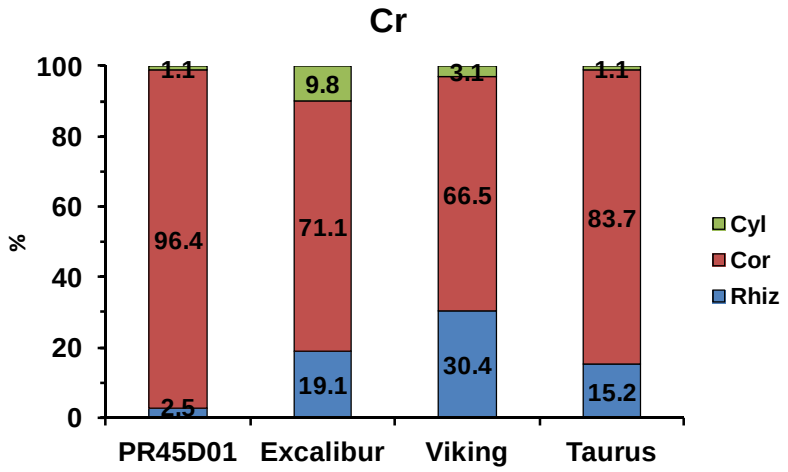

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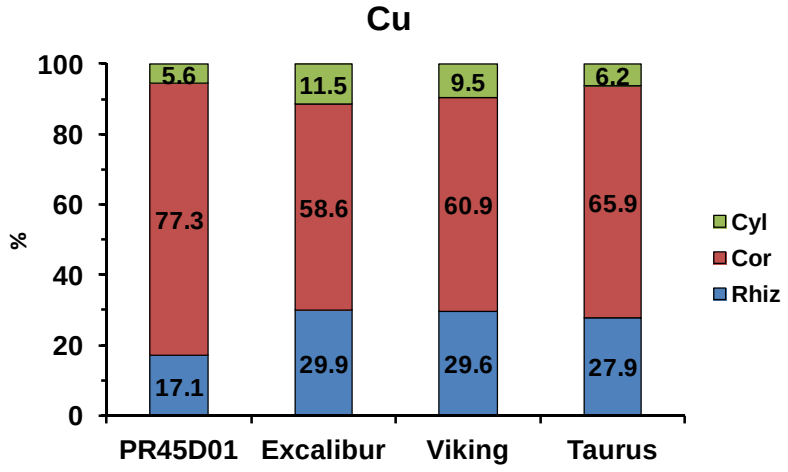

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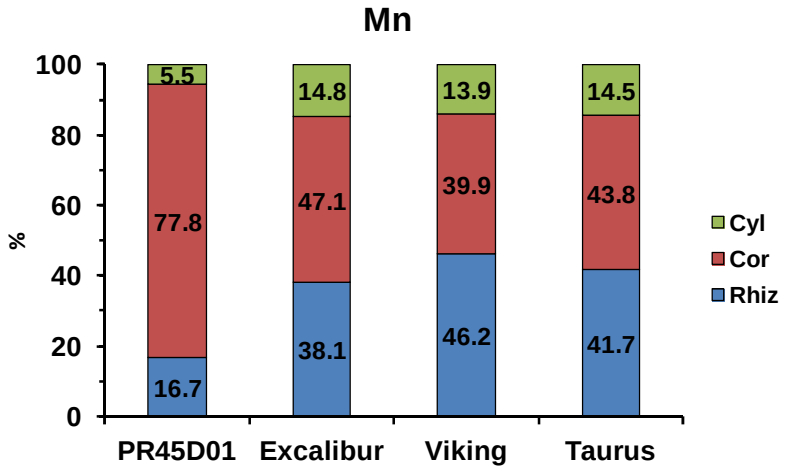

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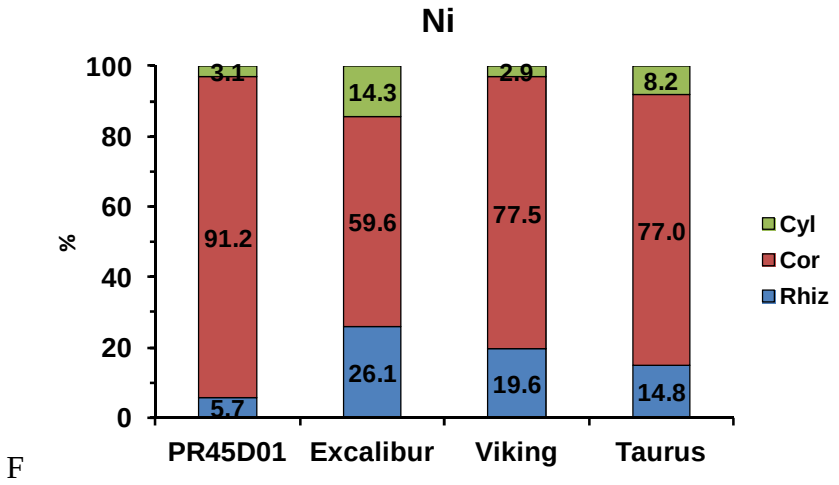

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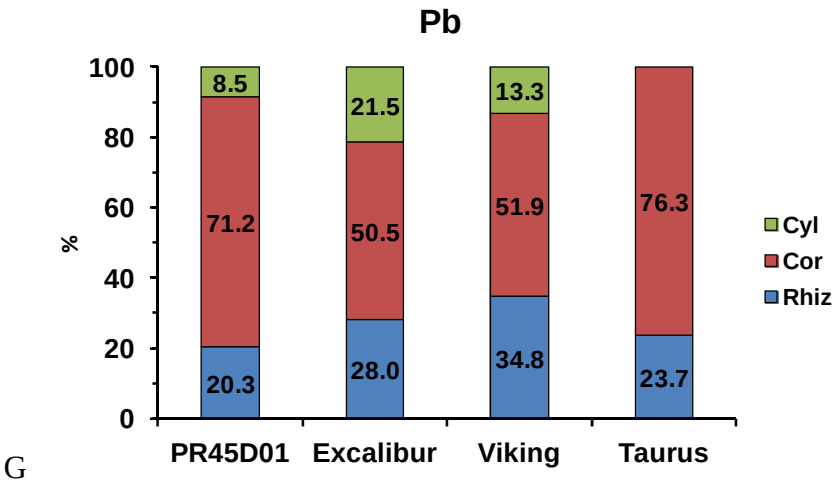

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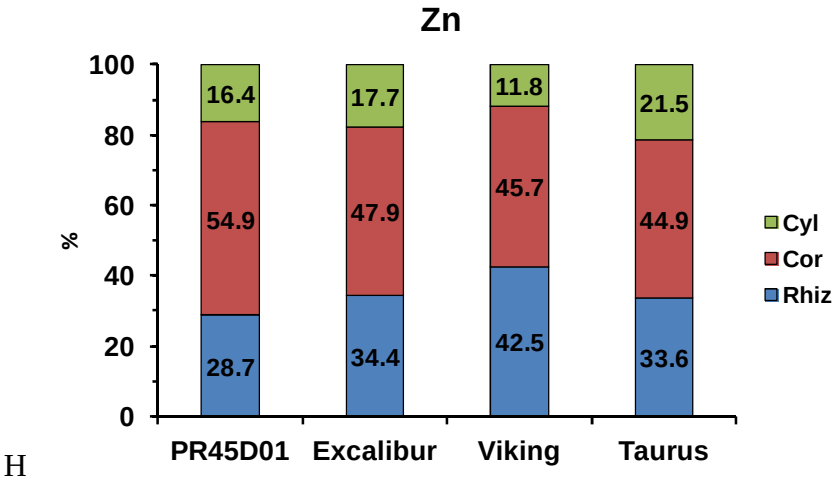

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Figure 15. Metal distribution (% out of content in mg per root) among different taproot tissues for the main effect "cultivar".

Despite the lower concentrations, the inner cortex (Cor) accumulated higher amount of metals due to its higher weight than the other tissues (Fig. 15). Metals had similar pattern of distribution among root tissues, with the distribution decreasing in the order Cor > Rhiz > Cyl. Nevertheless, the % of each metal in a specific tissue appeared generally different from those of the other metals, and apparently there were also differences in the distribution of the elements in different varieties.

1.3.5. Root breaking-down

Residual biomass during the degradation trial

The results for the residual root biomass for the main effect "cultivar" and "density" are reported in Tables 2 and 3 respectively.

Table 2. Percentage residual dry weight out of the initial value (%dw) for taproots of different cultivar. Values represent mean ± standard error for at each sampling date. Different letters indicate statistically significant differences (LSD test P ≤ 0.05).

Sampling	dw	PR45D01	Excalibur	Viking	Taurus
1	%	26.7 ± 2.93	30.9 ± 4.32	33.2 ± 2.81	29.17 ± 1.74
2	%	19.9 ± 2.76	21.4 ± 4.29	29.3 ± 5.74	25.6 ± 2.03
3	%	8.14 ± 2.23	7.13 ± 2.10	12.9 ± 4.93	7.00 ± 1.79
4	%	2.77 ± 1.12 (b)	5.24 ± 1.52 (ab)	11.4 ± 2.30 (a)	7.14 ± 2.20 (ab)

Table 3. Percentage residual dry weight out of initial value (%dw) of rapeseed taproots at different plant densities. Values represent mean ± standard error at each sampling date. Different letters indicate statistically significant differences (LSD test P≤ 0.05).

Sampling	dw	22 plants m^{-2}	44 plants m^{-2}	63 plants m^{-2}
1	%	32.8 ± 2.53	27.0 ± 2.15	30.1 ± 3.15
2	%	25.8 ± 3.44	21.6 ± 4.85	24.9 ± 1.34
3	%	9.28 ± 1.55	11.4 ± 3.73	5.74 ± 1.98
4	%	5.72 ± 1.20 (b)	9.63 ± 2.57 (a)	4.52 ± 0.91(b)

The % residual weight (%dw) was significant only at the last sampling, for both the main effects. PR45D01 had the lowest %dw and Viking the highest, followed by Taurus and Excalibur. The intermediate density allowed to conserve higher %dw than the other densities. The individual and per-hectare taproots weight were never significantly different among cultivar or densities during the degradation trial (data not shown).

Degradation trends for taproots

The taproot percentage residual dry weight (%dw) was used to assess the dynamic of organic substance degradation within the incubation timespan for both the main effects "cultivar" (Fig. 16) and "density" (Fig. 17).

The degradation patterns fitted a MMF model for both the main effects. The MMF model follows the general equation:

$$y = \frac{ab + cx^d}{b + x^d} \quad \text{(Eq. 1)}$$

Excalibur, Viking and Taurus were approximated through the same equation, and were therefore represented by the same curve, while PR45D01 followed a different trend, with a faster degradation compared to the other varieties (Fig. 16).

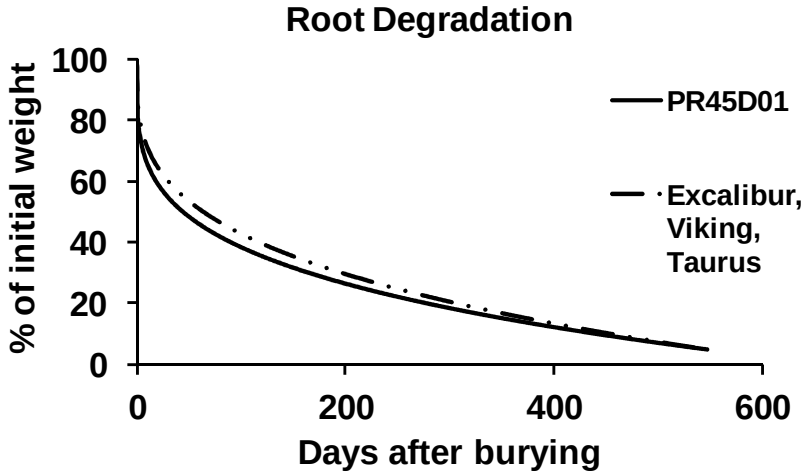


Figure 16. Predicted trends of root residual dry weight of degrading taproots (% out of initial dw) for different varieties. The model used was a MMF model [$y = (a \times b + c \times x^d)/(b + x^d)$].

The parameters for the MMF model describing root degradation for the main effect "cultivar" are reported in Table 4.

Table 4. MMF model parameters, coefficient of determination (R^2) and estimated half time ($t_{(1/2)}$) for taproot degradation of different rapeseed varieties.

Parameter	PR45D01	Excalibur	Viking	Taurus
a	9.9970E+01	9.9981E+01	9.9981E+01	9.9981E+01
b	1.4462E+07	2.9788E+05	2.9788E+05	2.9788E+05
c	-2.7341E+08	-4.2574E+06	-4.2574E+06	-4.2574E+06
d	2.5660E-01	3.0028E-01	3.0028E-01	3.0028E-01
R^2	0.9978	0.9992	0.9992	0.9992
t(1/2) (days)	45	65	65	65

The difference among PR45D01 and the other cultivar was particularly large at the beginning of the degradation process, and led to a estimated half-time for the loss of dry weight ($t_{(1/2)}$) of only 45 days, which is shorter than that estimated for the other cultivars (65 days).

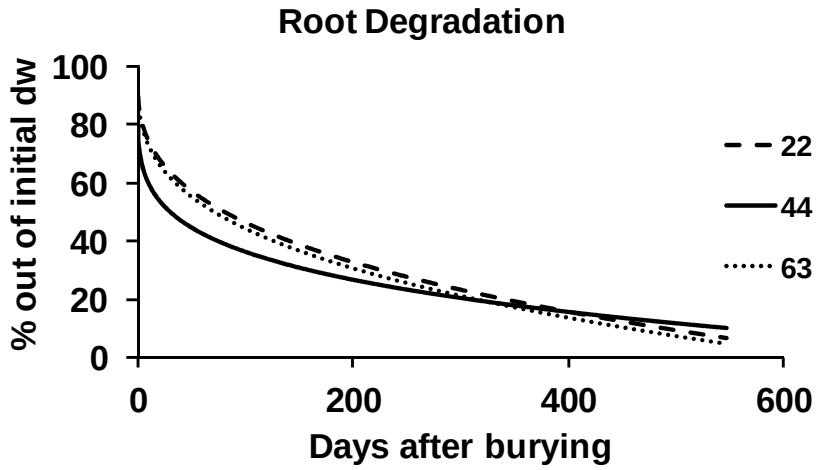


Figure 17. Predicted trends of root residual dry weight of degrading taproots (% out of initial dw) for different plant densities. The model used was a MMF model [$y = (a \times b + c \times x^d)/(b + x^d)$].

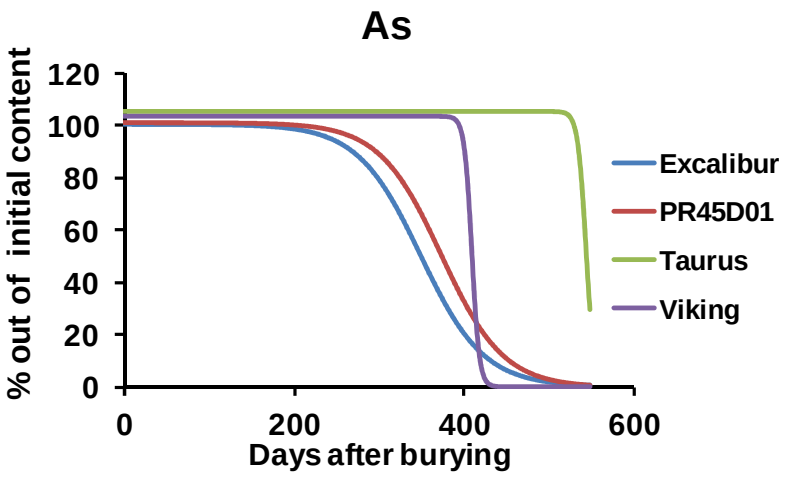
A specific trend for the residual dry weight of taproot was found for each plant density. The trends for the lowest and highest densities were very similar to each other (Fig. 17).

The parameters describing each equation are reported in Table 5.

Table 5. MMF model parameters, coefficient of determination (R^2) and estimated half time ($t_{(1/2)}$) for taproot degradation for different plant densities.

Parameter	22 plants m^{-2}	44 plants m^{-2}	63 plants m^{-2}
a	9.9944E+01	9.9989E+01	9.9939E+01
b	6.2375E+06	2.1509E+03	4.7699E+07
c	-7.4520E+07	-5.3882E+04	-6.2083E+08
d	3.2598E-01	2.0281E-01	3.1574E-01
R^2	0.99667	0.99901	0.99420
t(1/2) (days)	81	31	70

Heavy metals retention in degrading taproots for the main effect "cultivar"



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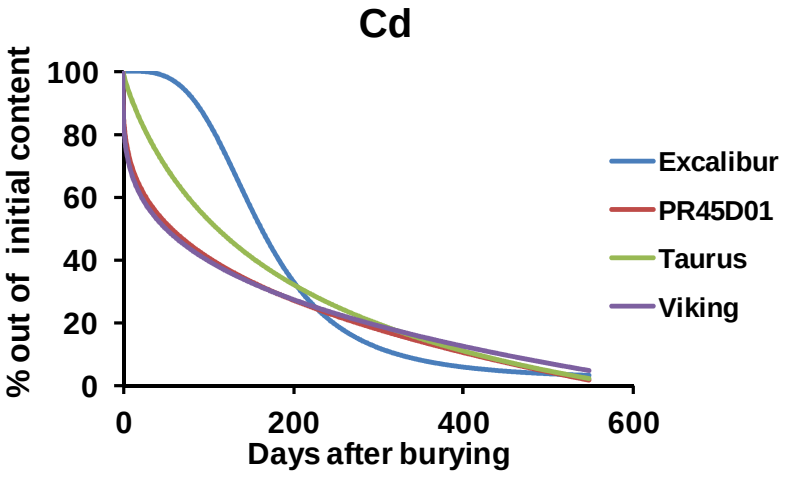

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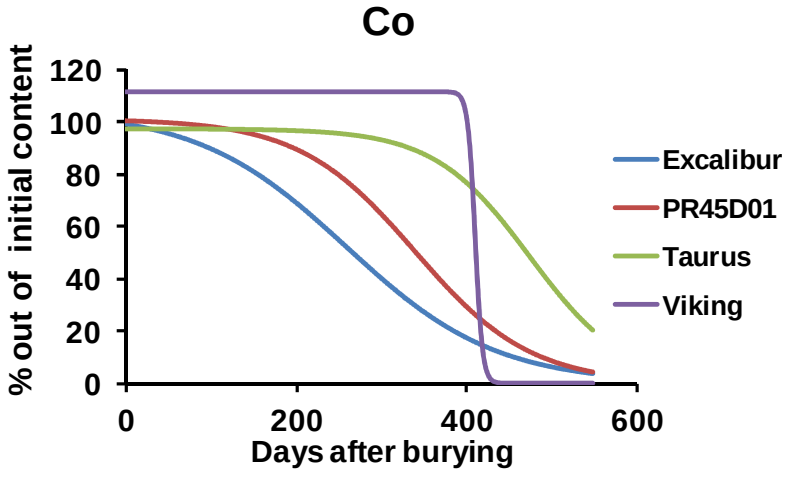

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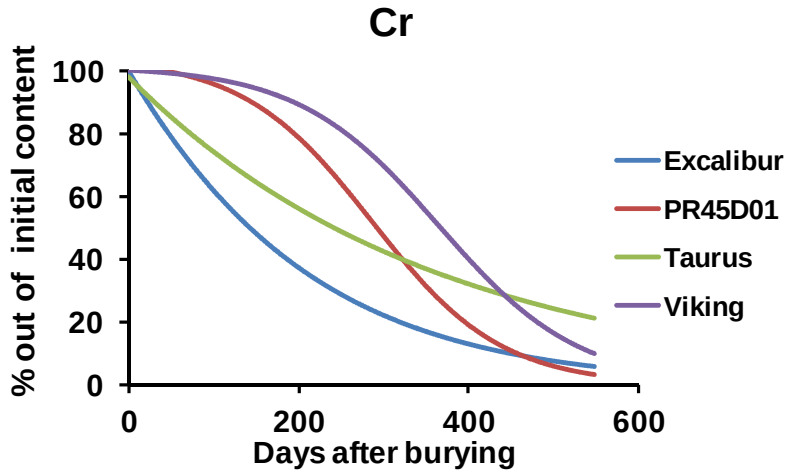

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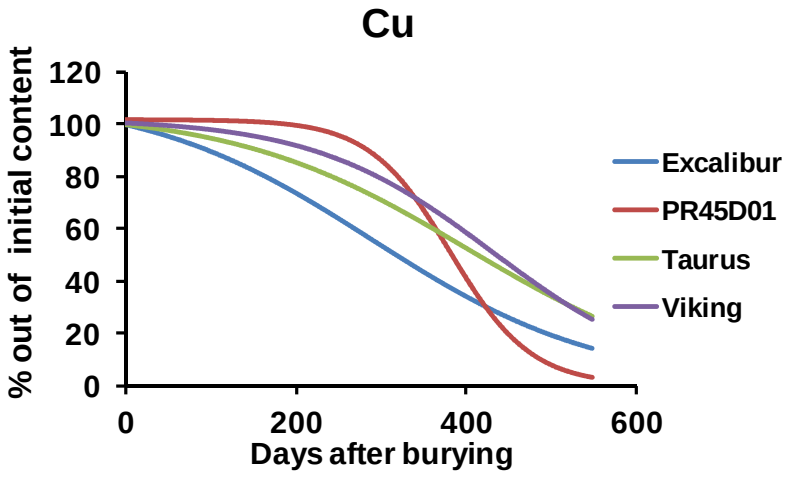

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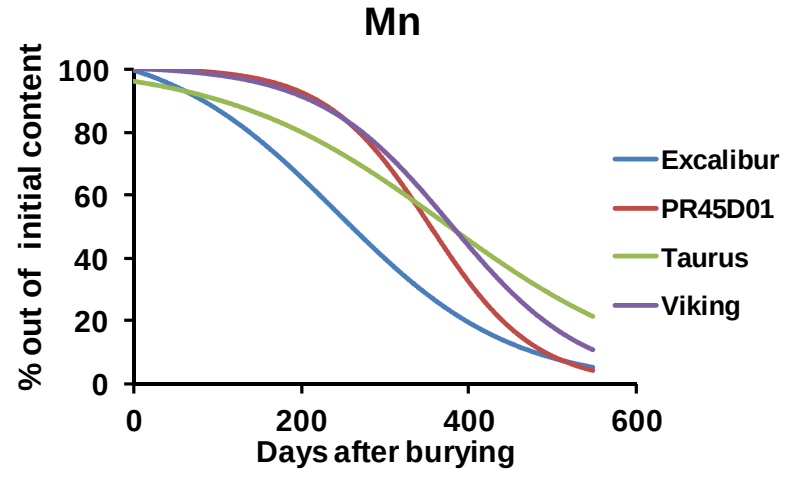

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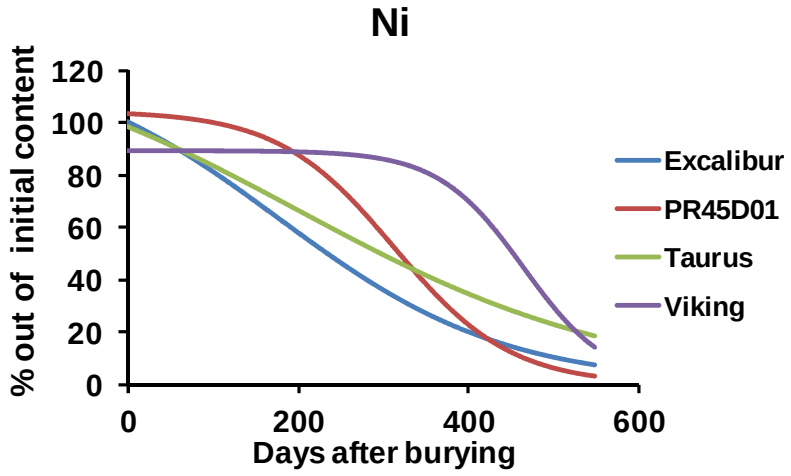

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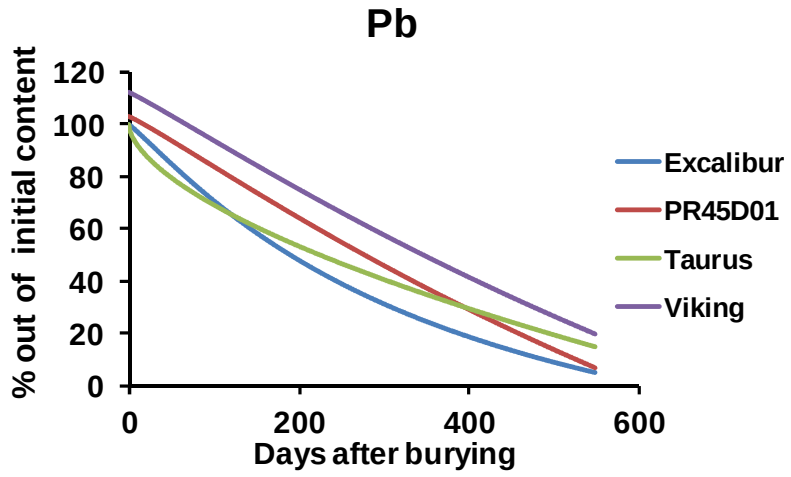

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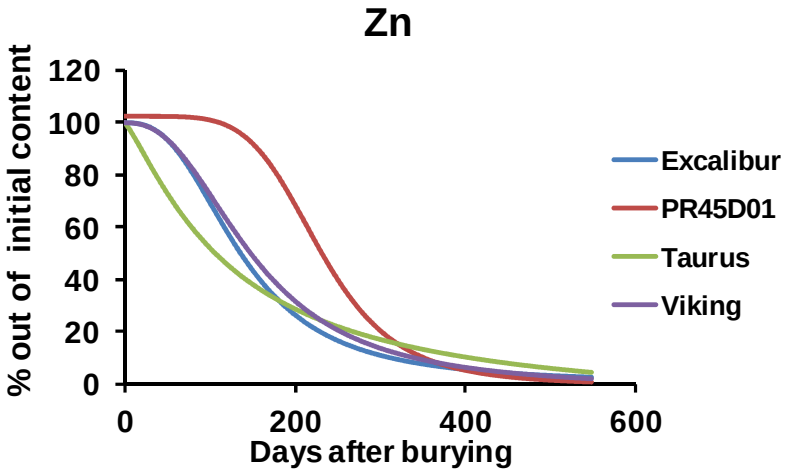

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Figure 18. Estimated trends for the residual metal content (% out of initial content expressed in g ha^{-1}) in rapeseed taproots for different varieties.

Table 6. Model parameters and coefficient of determination (R^2) for residual metal contents in rapeseed taproots for different varieties

Metal	Model	Parameter	PR45D01	Excalibur	Viking	Taurus
As	$a/(1+e^{(b-cx)})$ (Ratkovsky Model)	a	1.01E+02	1.00E+02	1.03E+02	1.05E+02
		b	-1.02E+01	-9.40E+00	-9.28E+01	-1.13E+02
		c	-2.75E-02	-2.70E-02	-2.27E-01	-2.07E-01
		R^2	0.9092	0.9777	0.8769	0.9895
Cd	$(ab+cx^d)/(b+x^d)$ (MMF Model)	a	1.00E+02	1.00E+02	1.00E+02	1.00E+02
		b	1.04E+06	4.13E+07	1.83E+07	1.15E+02
		c	-1.56E+07	1.87E+00	-3.19E+08	-4.17E+01
		d	2.98E-01	3.45E+00	2.69E-01	8.79E-01
		R^2	0.9945	0.9989	0.9972	0.9966
Co	$a/(1+e^{(b-cx)})$ (Ratkovsky Model)	a	1.01E+02	1.05E+02	1.12E+02	9.73E+01
		b	-4.99E+00	-2.95E+00	-9.52E+01	-8.43E+00
		c	-1.47E-02	-1.14E-02	-2.33E-01	-1.78E-02
		R^2	0.9886	0.9922	0.8781	0.9914
Cr	$a/(1+e^{(b-cx)})$ (Ratkovsky Model)	a	1.04E+02	6.43E+02	1.01E+02	1.19E+04
		b	-3.77E+00	1.69E+00	-4.43E+00	4.79E+00
		c	-1.32E-02	-5.48E-03	-1.22E-02	-2.80E-03
		R^2	0.9935	0.9967	1	0.8773
Cu	$a/(1+e^{(b-cx)})$ (Ratkovsky Model)	a	1.02E+02	1.12E+02	1.02E+02	1.05E+02
		b	-8.05E+00	-2.16E+00	-4.09E+00	-2.93E+00
		c	-2.11E-02	-7.46E-03	-9.50E-03	-7.32E-03
		R^2	0.9685	0.9995	0.9964	1
Mn	$a/(1+e^{(b-cx)})$ (Ratkovsky Model)	a	1.01E+02	1.09E+02	1.01E+02	1.02E+02
		b	-5.68E+00	-2.35E+00	-4.77E+00	-2.80E+00
		c	-1.61E-02	-9.68E-03	-1.26E-02	-7.55E-03
		R^2	0.9837	0.997	0.9951	0.9584
Ni	$a/(1+e^{(b-cx)})$ (Ratkovsky Model)	a	1.04E+02	1.27E+02	8.92E+01	1.33E+02
		b	-4.59E+00	-1.34E+00	-9.28E+00	-1.05E+00
		c	-1.47E-02	-7.56E-03	-2.00E-02	-5.24E-03
		R^2	0.9873	0.9833	0.9023	0.9547
Pb	$(ab+cx^d)/(b+x^d)$ (MMF Model)	a	1.03E+02	1.00E+02	1.12E+02	9.95E+01
		b	2.78E+03	7.38E+02	2.84E+03	3.41E+08
		c	-2.45E+02	-5.86E+01	-2.58E+02	-6.67E+08
		d	1.10E+00	1.11E+00	1.09E+00	5.98E-01
		R^2	0.9793	0.9998	0.8829	0.9696
Zn	$(ab+cx^d)/(b+x^d)$ (MMF Model)	a	1.02E+02	1.00E+02	1.00E+02	1.00E+02
		b	1.43E+12	5.25E+05	2.38E+05	4.17E+02
		c	-7.26E-01	3.90E-01	-1.90E+00	-1.07E+01
		d	5.15E+00	2.68E+00	2.47E+00	1.25E+00
		R^2	0.995	1	0.9999	0.9992

The estimated trends for residual metal contents in rapeseed taproots for different cultivar are reported in Fig. 18, whereas the parameters for each equation are reported in Table 6.

Metal contents fitted either a Ratkowsky (As, Co, Cr, Cu, Mn, Ni) or a MMF (Cd, Pb and Zn) model, both belonging to the family of sigmoidal models. Nevertheless, metal contents followed different patterns depending on the cultivar. Viking seemed to release metals (As, Co, Cr, Mn, Ni) more slowly than the other cultivar. A similar behaviour was found for Taurus (As, Co, Cu, Mn). On the contrary, PR45D01 seemed more effective at retaining Zn. Excalibur resulted the most effective for Cd, but generally had faster releasing of metals (Co, Cu, Mn, Ni) compared with the other cultivars.

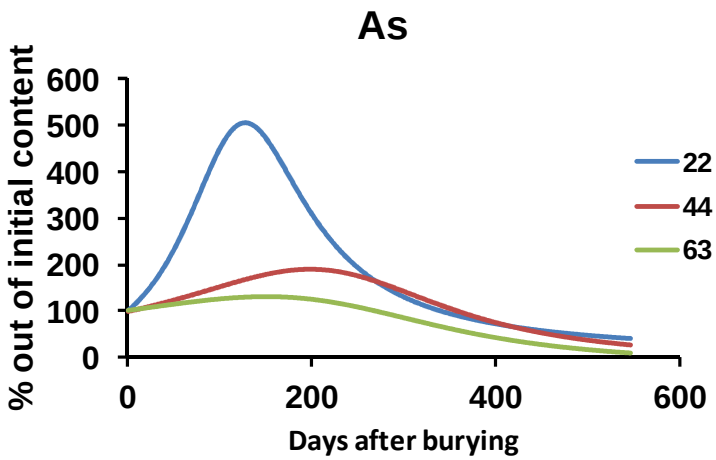
Sometimes (As × Viking, As × Taurus, Co × Viking, Ni × PR45D01 and Pb × Viking), the estimated trends resulted in initial percentage higher than 100%.

Table 7. Estimated half time for the residual metal content in rapeseed taproots of different varieties.

Metal	$t_{(1/2)}$	PR45D01	Excalibur	Viking	Taurus
As	Date	11/06/2010	17/05/2010	17/07/2010	28/11/2010
	Days after burying	373	348	409	543
Cd	Date	29/07/2009	12/11/2009	23/07/2009	21/09/2009
	Days after burying	56	162	50	110
Co	Date	09/05/2010	24/02/2010	18/07/2010	16/09/2010
	Days after burying	340	266	410	470
Cr	Date	22/03/2010	23/10/2009	04/06/2010	29/01/2010
	Days after burying	292	142	366	240
Cu	Date	20/06/2010	16/04/2010	11/08/2010	22/07/2010
	Days after burying	382	317	434	414
Mn	Date	23/05/2010	18/02/2010	18/06/2010	15/06/2010
	Days after burying	354	260	380	377
Ni	Date	17/04/2010	22/01/2010	28/08/2010	27/03/2010
	Days after burying	318	233	451	297
Pb	Date	05/03/2010	09/12/2009	15/05/2010	11/01/2010
	Days after burying	275	189	346	222
Zn	Date	20/01/2010	17/10/2009	29/10/2009	17/09/2009
	Days after burying	231	136	148	106

The estimated $t_{(1/2)}$ for metals for the main effect “cultivar” (Table 7) were different among the varieties, and reflect the different ability at retaining metals. Noticeable, the half time were always longer than those estimated for root biomass.

Heavy metal retention in degrading taproots for the main effect "density"



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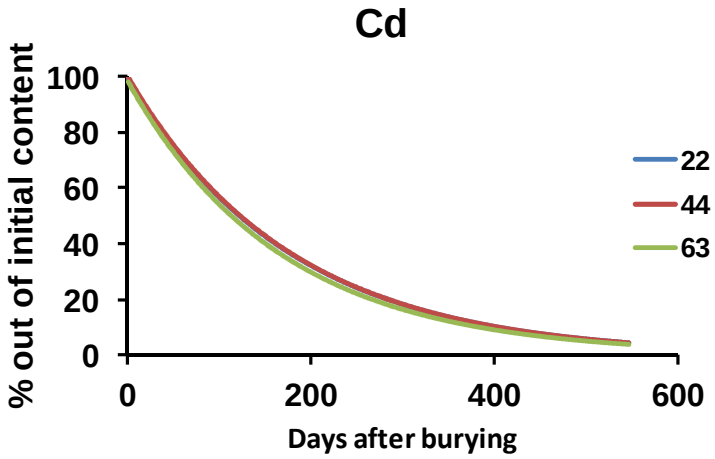

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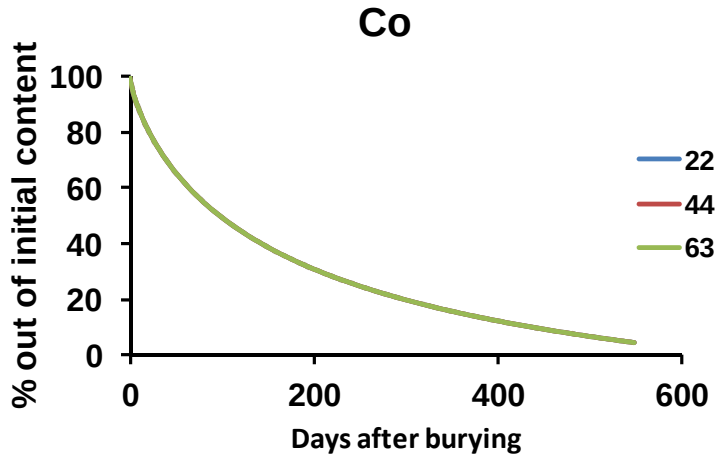

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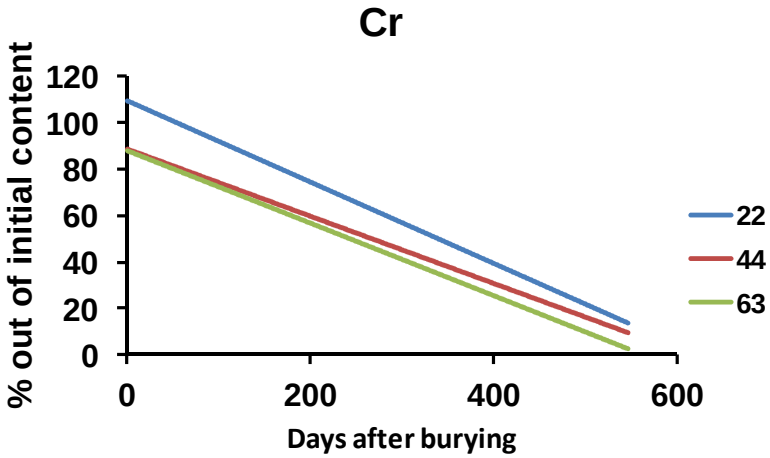

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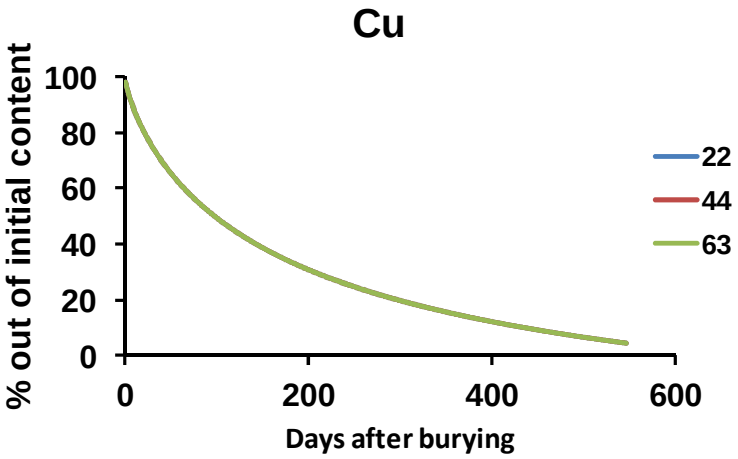

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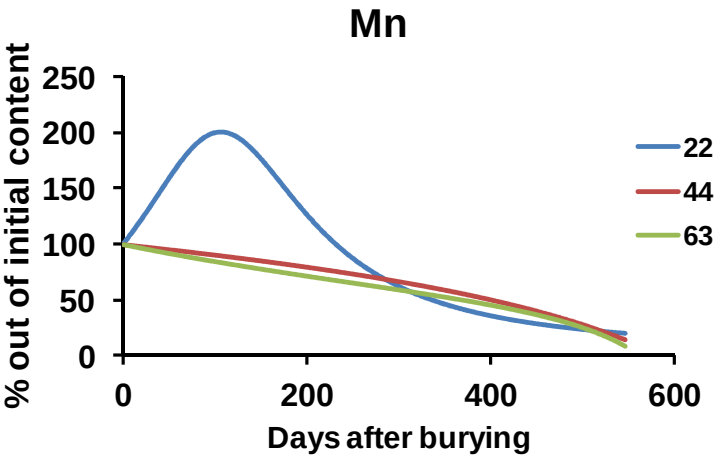

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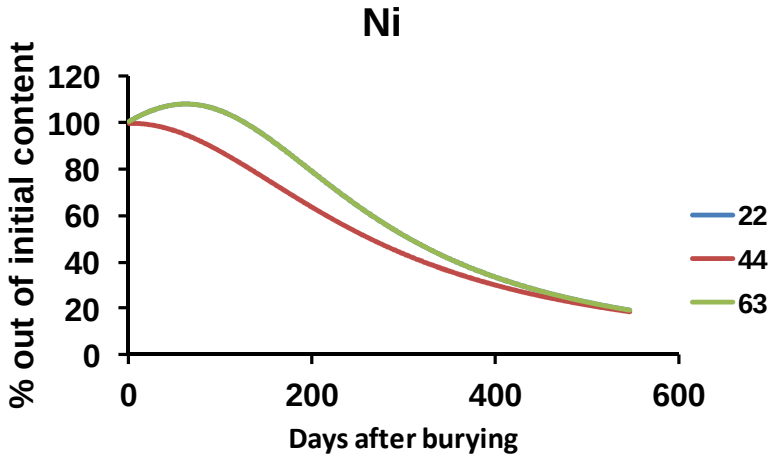

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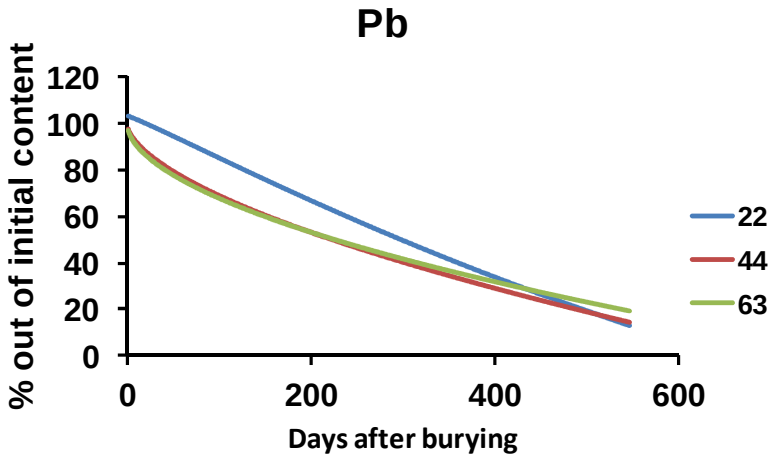

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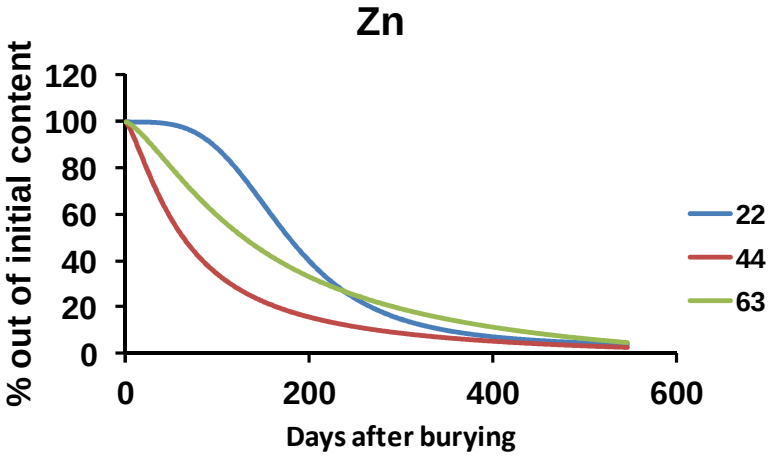

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Figure 19. Estimated trends for the residual metal content (% out of initial content expressed in g ha^{-1}) in rapeseed taproots at different plant densities.

Table 8. Model parameters and coefficient of determination (R^2) for residual metal contents in rapeseed taproots at different plant densities.

Metal	Model	Parameter	22	44	63
As	(a+b*x)/(1 + c*x + d*x^2) (Rational Model)	a	1.00E+02	9.95E+01	1.00E+02
		b	5.20E-01	-9.31E-02	-1.51E-01
		c	-1.15E-02	-5.28E-03	-4.18E-03
		d	4.89E-05	1.21E-05	9.96E-06
		R^2	0.9957	0.9927	1.0000
Cd	a*exp(b*x) (Exponential Model)	a	9.98E+01	1.00E+02	9.89E+01
		b	-5.65E-03	-5.64E-03	-6.00E-03
		R^2	0.9981	0.9984	0.9763
Co	(a*b + c*x^d)/(b + x^d) (MMF Model)	a	1.00E+02	1.00E+02	1.00E+02
		b	6.73E+01	6.73E+01	6.73E+01
		c	-3.93E+01	-3.93E+01	-3.93E+01
		d	7.93E-01	7.93E-01	7.93E-01
		R^2	0.9983	0.9983	0.9983
Cr	a + b*x (Linear Regression)	a	1.09E+02	8.89E+01	8.78E+01
		b	-1.75E-01	-1.46E-01	-1.56E-01
		R^2	0.8881	0.9016	0.9007
Cu	(a*b + c*x^d)/(b + x^d) (MMF Model)	a	1.00E+02	1.00E+02	1.00E+02
		b	6.73E+01	6.73E+01	6.73E+01
		c	-3.93E+01	-3.93E+01	-3.93E+01
		d	7.93E-01	7.93E-01	7.93E-01
		R^2	0.9983	0.9983	0.9983
Mn	(a+bx)/(1+cx+dx^2) (Rational Model)	a	1.00E+02	1.00E+02	1.00E+02
		b	1.63E-01	-1.71E-01	-1.78E-01
		c	-8.59E-03	-8.09E-04	-7.51E-05
		d	4.41E-05	-2.69E-07	-2.08E-06
		R^2	0.9868	1.0000	0.9995
Ni	1/(a + b*x + c*x^2) (Reciprocal Quadratic)	a	9.98E-03	1.00E-02	9.98E-03
		b	-2.26E-05	-9.46E-07	-2.26E-05
		c	1.80E-07	1.45E-07	1.80E-07
		R^2	0.9708	0.9591	0.0000
Pb	(ab+cx^d)/(b+x^d) (MMF Model)	a	1.03E+02	9.98E+01	9.98E+01
		b	2.74E+03	1.27E+08	5.92E+07
		c	-2.42E+02	-2.42E+08	-1.53E+08
		d	1.09E+00	6.03E-01	5.46E-01
		R^2	0.9782	0.9876	0.9061
Zn	(ab+cx^d)/(b+x^d) (MMF Model)	a	1.00E+02	1.00E+02	1.00E+02
		b	1.22E+08	2.99E+02	1.16E+03
		c	2.55E+00	-3.19E+00	-1.05E+01
		d	3.60E+00	1.36E+00	1.41E+00
		R^2	0.9956	1.0000	0.9990

Generally, each metal was retained at different extent from rapeseed taproots at different plant densities (Fig. 19), as visible from the parameter reported in Table 8.

Co and Cu followed the same retention trend for all the densities (Fig. 19C and 19E), whereas Ni followed the same trend for the highest and lowest densities (Fig.19G). Cd followed very similar trends among the different densities (Fig. 19B).

For most metals (As, Cr, Pb and Zn), the retention pattern appeared slower at 22 plant m^{-2} (Fig. 19A, 19D, 19H, 19I); however, the intermediate density appeared to release Mn more slowly than the other densities (Fig. 19F), and it was as effective as the lowest density for As, but had the worst retention of Zn. The highest density was as effective as the lowest density at retaining Ni and as effective as the intermediate density for Pb, but had the worst retention ability for As and Cr.

For As, Mn and Ni, the % content increased for the lowest density at the beginning of the degradation of roots, while this effect was not observed in the other density or for the main effect "cultivar".

Table 9. Estimated half time for the residual metal in rapeseed taproots at different plant densities.

Metal	$t_{(1/2)}$	22	44	63
As	Date	05/10/2010	07/09/2010	18/06/2010
	Days after burying	489	461	380
Cd	Date	03/10/2009	04/10/2009	25/09/2009
	Days after burying	122	123	114
Co	Date	09/09/2009	09/09/2009	09/09/2009
	Days after burying	98	98	98
Cr	Date	08/05/2010	24/02/2010	30/01/2010
	Days after burying	339	266	241
Cu	Date	08/08/2009	08/08/2009	08/08/2009
	Days after burying	97	97	97
Mn	Date	05/05/2010	08/07/2010	07/06/2010
	Days after burying	336	400	369
Ni	Date	06/04/2010	23/02/2010	06/04/2010
	Days after burying	307	265	307
Pb	Date	27/03/2010	13/01/2010	14/01/2010
	Days after burying	297	224	225
Zn	Date	28/11/2010	04/08/2009	09/10/2009
	Days after burying	178	62	128

The equations describing the residual content of each metal allowed to estimate the $t_{(1/2)}$ for metal contents (Table 9). The $t_{(1/2)}$ were generally longer than those of the residual biomass, as happened for the main effect "cultivar".

Heavy metal proportions in degrading taproots

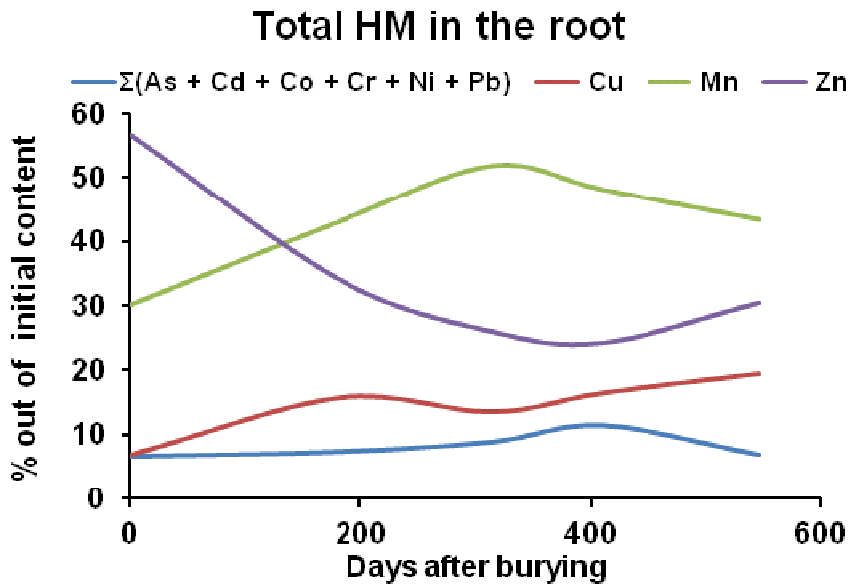

Figure 20. Relative content of metals (% out of the total amount of metals expressed in mmol per root) irrespective of the cultivar and density.

The total composition in heavy metals of the taproots indicate that the proportion of each metal changes during the degradation (Fig. 20). Some elements (Mn, Cu) become relatively more concentrated, and other (Zn) become less concentrated over time.

1.4. Discussion

1.4.1. Metal removals through aboveground biomass and taproots

Biomass, along with metal concentrations in the shoots and roots, is one of the main parameters to take into account to estimate plants' ability at accumulating metals (Li *et al*., 2003; Quartacci *et al*., 2006).

Rapeseed might be effective for the management of heavy metal-polluted soils, due to its ability at removing metals thorough the above and below -ground biomass. In fact, metal removals were generally high (1 kg ha^{-1}), and increased with higher plant densities.

Among the genotypes tested here, all the hybrids produced larger aboveground biomass than the variety, and removed higher amounts of metals, confirming their higher potential for phytoextraction. For phytostabilization in taproots, the variety was overall as effective as the hybrids. Nevertheless, the differences in the removals of specific elements (Cd, Co, Cr, Cu, Ni, Pb) through the taproot of different cultivars, suggest that the intraspecific variability should be investigated to improve the effectiveness of phytostabilization.

Here, Taurus resulted the best cultivar for metal accumulation in the aboveground biomass, and should be preferred even for phytostabilization in taproots, in view of its better performance for the removals of most metals.

Metal accumulation in shoots and roots had different responses to the increase in sowing density, and in rapeseed phytoextraction is more easily enhanced at increasing density than phytostabilization. However, the results indicate that agronomic factors (i.e. plant density and choice of the genotype) can contribute increasing both metal phytoextraction and, to a lesser extent, phytostabilization, suggesting that the efficiency of green technologies can be improved through proper agronomic practices.

1.4.2. Root degradation in soil and effectiveness of phytostabilization of metals

The decomposition of leaf-derived litter is affected by its physical-chemical composition (Cornelissen, 1996), and this is true for litter in general. Many indices, including the concentration of lignin, can be related to the process of mineralization of organic matter (Heal *et al.*, 1997), and it is therefore reasonable to retain that other chemical compounds relatively recalcitrant to degradation (i.e. cellulose) might be useful to determine the degradation pattern of plant-derived organic matter. However, the chemical composition can not explain the whole process of plant litter

degradation (Paustian *et al*., 1997), since environmental parameters (i.e. temperature, moisture) are also involved in determining it (Singh and Gupta, 1977). However, in this study, only the chemical composition of taproots (i.e., content of fibers) was taken into account for determining the degradation pattern of rapeseed taproots, since the pedo-climatic conditions where the same for all the treatments.

Since the chemical composition (cellulose and lignin) was the same irrespective of plant density, no differences in the degradation patterns were expected for the main effect "density", and the residual biomass (kg ha^{-1}) was expected to be higher at 63 plant m^{-2}. For cultivars, no hypothesis could be built up depending on the content of fiber, since the contents of lignin and cellulose were not measured for the varieties.

On the contrary, for different taproot tissues, different degradation dynamics were expected, because of the different chemical composition: the inner cortex (Cor) was expected to be the most recalcitrant tissue to degradation in view of its higher content of fibers, while the inner cylinder (Cyl) was expected to be the most easily degradable.

The results of the degradation trial (Fig. 16 and 17) suggest that taproot are degraded with different dynamics depending on the genotype and sowing density, although these differences generally did not lead to significant differences in the residual biomass in the long term. Different degradation trends for the main effect "density" were therefore not consistent with the results for the content of fiber; this suggests that the content of lignin and cellulose can not explain the degradation patterns of taproots within the same species, and therefore other variables might be more important for determining intraspecific differences in the process.

However, as expected, different root tissues were degraded with different rates, with Cor being the most resistant tissue to degradation, confirming that the content of lignin and cellulose might be good predictors for the degradability of plant residues (i.e. different tissues).

Overall, our view is that small differences in the degradation patters might exist among densities and cultivar, due to differences in root size. The size (diameter) affects the mean lifespan of roots (Tierney and Fahey, 2001; 2002; Van Der Krift and Berendse, 2002), and it might aslo affect the degradation dynamics of dead roots.

However, overall the residual biomass (kg ha^{-1}) available for metal retention in the long term can be considered independent from the density and genotype.

As regard the model proposed for the dynamic of taproot degradation, the MMF model (sigmoidal family) resulted the best choice for approximating the % residual biomass for the main effects "cultivar" and "density", and in general mirrored the experimental data, as confirmed by the high coefficients of determination. The results therefore suggest that the MMF model is suitable for describing rapeseed taproots degradation in general, irrespective of the cultivation factors, at least in this soil. However, it is likely that degradation of litter might be modeled through other models too, depending on the species (Henriksen and Breland, 1999) and quality of litter (Swift *et al*., 1979). For example, Curry and Byrne (1997) found that both an exponential decay and a linear model gave good fitting for the loss of weight in winter wheat over time. Therefore, it is likely that the specific characteristics of both the litter and pedo-climatic condition might greatly affect the degradation rate of plant litter.

The experimental data and the shape of the curves suggest that the degradation of root was very quick at the beginning of the degradation trial, as confirmed by the very short $t_{(1/2)}$ estimated for both the cultivars and densities. The initial loss of organic substance might be due to mineralization of the readily degradable compounds, especially in the rhizoderm and inner cylinder; then, it is likely that, when only the inner cortex remained, the degradation slowed down.

However, the degradation of rapeseed taproots is almost completed in 18 months. Nevertheless, since after 18 months there still was about 10% of not-degraded biomass, we retain that in the long term it is possible to increase the organic substance for metal stabilization in soil by adding plant residues from the following cultivation cycle, thus increasing the effectiveness of *in-planta* phytostabilization.

Finally, the nylon net bags might have significantly affected the rate of root decomposition, therefore it is likely that the trends found here are biased and not representative of the real degradation occurring in the field. In fact, coarse mesh allows earthworms and other macroinvertebrates to contribute to the degradation of litter and to accelerate the mineralization rate, wheres when the size of the mesh does not allow macroinvertebrates to stay in contact with the litter, the degradation results

significantly slower (Curry and Byrne, 1997). However, since all the data were affected by the same bias, the results remain valid for the evaluation of the influence of the factors under study.

1.4.3. Heavy metal retention in taproots

Heavy metal residual contents in breaking down taproots depend on their initial concentrations and the releasing into the soil as a consequence of root degradation . The initial concentrations in turn depend on roots ability at absorbing metals and retaining instead of translocating them to the shoots, and this ability is different according to the metal and the plant species: for instance, many *Brassicaceae* have good ability at absorbing and accumulating metals in both shoots and roots (Vamerali *et al*., 2012).

The results of the degradation trail confirmed that *B. napus* might be suitable for phytostabilization, especially for Cu and Zn, that were accumulated at higher extent than other metals.

In taproots, metals were mainly accumulated in the inner cortex, which was the more recalcitrant tissue to degradation. This explains the slower loss of metals than biomass from degrading taproots, and suggests that metal retention in taproots is effective despite the loss of organic matter. In addition, the differences in degradation patter ns of different taproot tissues might explain the differences in the retention patterns for different metals (Fig.18) and the changes in the composition in heavy metals in the roots during the degradation (Fig. 20): in fact, those metals that were found mainly in the inner cortex (Cr, Ni, Pb) were released more slowly than those stored at relatively high percentages in the inner cylinder or in the external cortex (Cd, Zn).

Metal retention was different depending on the variety, and this is consistent with the observation that the same element had different distribution in root tissues for different cultivars. Cultivars with higher relative contents of metals in the Cor, where therefore expected to be more effective at retaining metals. However, the relative distribution of metals in root tissues was only seldom consistent with the trend for metal releasing we found for different cultivar. For Zn and Cu it was found

a perfect match between metal distribution in root tissues depending on the cultivar and the releasing trend, while for other metals there was only a partial match between the expectation and the retention trends found: in general, higher contents in the Cor were associated to slower releasing at the beginning of the degradation in PR45D01 (Cr, Cu, Mn and Ni), but for Viking (Co, Cr, Mn, Pb) and Taurus (Cd, Pb) often the releasing of metals did not seemed related to the relative contents in the Cor. It is likely that when significant proportion of metal are stored in the other tissues than the Cor, the overall trend for metal retention is more difficult to predict referring only to the relative distribution of the element among the root tissues.

Higher densities were initially supposed to allow maintaining significantly higher metal contents over time, due to the higher initial accumulation, but in the end this hypothesis was rejected. However, at higher densities the initial accumulation of metals in taproots is larger, thus resulting more effective for phytostabilization.

The increase in metal contents during taproot degradation observed for the main effect "density" were attributed to the ability of organic matter to adsorb HM from the soil solution. This suggest that stabilization might be enhanced irrespective of the initial metal stock.

Overall, the results suggest that metal distribution in root could be useful to describe patterns of releasing from breaking down roots, but other factor must be involved in this process, and it is likely that more reliable estimation trends for metal retention might be obtained considering other chemical characteristics of the roots and soil.

1.5. Conclusions

Root degradation is a complex process that here was described considering only the time span between burying and sampling date and the content of fibers in taproots. Therefore the patterns of degradation and metal releasing are biased by the simplification of the process, but general conclusion could be drawn.

In the long term, the amount of recalcitrant biomass available for metal retention is independent from both the genotype and sowing density, therefore for an effective phytoextraction and phytostabilization the varieties producing higher

biomass (Taurus) should be preferred to those that produce smaller biomass, since higher biomass allows to accumulate higher amounts of metals. For the same reason, high densities should be preferred.

Irrespective of the sowing density and genotype, the degradation of organic matter occurs quickly, but in the long term (18 months) there is still about 10% of organic matter which retain metals. Therefore, phytostabilization in taproots is feasible, and the amount of metals retained in degrading taproots can be increased yearly by adding litter from the following cultivation cycle.

The patterns of metal releasing depends on the distribution of metals in the taproot tissues, the metal itself the genotype, and the chemical composition of the taproot tissues. However, these variables are not able to completely explain the degradation of taproots and metal releasing patterns, and clearly further research is needed to clear the role of different variables in determining the rate of taproot degradation.

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Chapter 2

Dynamics of root degradation and metal release with *in planta* phytostabilization: effects of different soil contamination levels

Abstract

Metal stabilization in plant roots is an emerging technology for the remediation of polluted soil which aims at immobilizing metals, possibly in the long-term, making them unavailable for organisms. However, when roots are degraded as a consequence of soil microbial activity, the metals previously stored are again released into the soil. In this study, it was assessed the time span within pollutants are retained in the taproot of *Brassica napus* L. var. *oleifera* under different soil pollution levels. The roots were buried in either metal-polluted or unpolluted- soil-filled boxes at 10 cm depth and collected periodically to measure the residual weight and metal contents. Root degradation trend were approximated through a sigmoidal model, and the estimated half-time for root biomass was 154 day in the metal-polluted soil and 114 in the unpolluted one. The slower root degradation under soil contamination was attributed to a lower microbial activity, as revealed by the soil mean fluoroscein content (0.99 ± 0.07 vs. 0.73 ± 0.07) over the whole period of incubation. Nevertheless, metal concentration in degrading roots was found to increase over time, especially in the polluted soil, probably due to metal adsorption onto organic matter. Adsorption was surely enhanced by the high metal bioavailability of the polluted soil and by water stagnation in the boxes of both treatments as a consequence of increased metal solubilization after rains.

It is concluded that phytostabilization with *B. napus* is a feasible phytomanagement option in metal-polluted soil only if new litter is yearly provided through new cultivation cycles, in order to increase the organic matter pool. It also seems that the effectiveness of metal immobilization might be improved by preventing metal leaching through various means (e.g., impermeable barriers).

2.1. Introduction

Heavy metals are common contaminants in industrial and agricultural soils, and reclamation of contaminated areas is needed to reduce human exposure to toxic elements through the direct contact with polluted soil and through the food chain (Robinson *et al*., 2009). Besides the traditional reclamation techniques, green technologies exploiting higher plants for soil remediation (collectively referred to as phytoremediation) have been recently developed, and nowadays many plant-mediated techniques are available for soil remediation (Zerbi and Marchiol, 2004).

One of the green technologies potentially useful for soil reclamation is the stabilization of heavy metals in the soil; the plant-mediated stabilization is referred as phytostabilization, and aims at immobilizing heavy metals in the rhizosphere, making them unavailable for soil organisms and thus preventing the food-chain contamination rather than at removing metals from the soil. Phytostabilization can occur either in the soil (*ex-planta* stabilization) or in the roots (*in-planta* stabilization). The first occurs through changes in metal bioavailability and solubility through modification in pH and precipitation or adsorption onto soil particles; for instance, bioavailability of heavy metals can be effectively reduced by rising the soil pH (McGrath, 1998; Salt *et al*., 1998) or through adsorption onto soil organic matter (Cunningham *et al*., 1996). *In planta* stabilization occurs through accumulation of heavy metals in plant roots, so that metals can not enter the food chain, because they are not transferred to the harvestable biomass (Robinson *et al*., 2009). Unfortunately, phytostabilization in plant roots is only a short-lived solution for soil pollution, because roots are degraded by soil microorganisms (e.g., bacteria, fungi), and the elements previously accumulated are therefore released into the soil (Vangronsveld *et al*., 1995). Nevertheless, there is not information available in the literature about the relation between the degradation of plant material and the releasing of heavy metals, so that the rate of metal releasing from plant material is still unknown.

In a previous experiment, it was found that rapeseed tap roots are completely degraded in about 18 months, but metal release is slower than the loss of biomass, because metals are mainly stored in the most recalcitrant tissues (i.e., internal cortex). As a result, despite the estimated half time for root biomass in absence of contamination was only 65 days, those of metals ranged from about 100 to 420 days,

depending on the element, with most metals having a half time > 300 days. In this trial it was also found that root biomass and the metal stock had a different estimated half-time in different cultivars, suggesting the importance of genotype choice.

In this study the root degradation experiment with *Brassica napus* L. var. *oleifera* was repeated, with the objective to assess the rate of root degradation and metal release over time in relation to the level of soil pollution. The effects of some environmental variables, such as soil temperature and moisture on litter degradation in soils have been already studied in the past (e.g., Henriksen and Breland, 1999a), but there is lack of information on the influence of soil pollution.

2.2. Material and Methods

The trial was carried out at the experimental farm "Lucio Toniolo" of the University of Padova (Italy), and the analysis were performed at Department of Agriculture, Food, Natural resources, Animals and Environment of the same University.

2.2.1. Experimental set up for cultivation

Two cultivars of *Brassica napus* L. var. *oleifera*, i.e. PR45D01 (semi-dwarf hybrid, Pioneer) and Excalibur (CHH hybrid, Dekalb) were sown in the silty-loam soil of the experimental farm of Padova University on September 30 2010. With the aim to studying the effects of seed density on root growth and metal accumulation, rapeseed was grown at 44 and 63 plants m^{-2} within a split plot design with 3 replicates (4.5 ×12 m plot size). After ploughing and harrowing, the soil was fertilized with 60 kg ha^{-1} of P_2O_5 as triple perphosphate, and 60 kg ha^{-1} of K_2O as potassium sulphate. Nitrogen was supplied in spring time at the dose of 100 kg ha^{-1} as ammonic sulfate followed by a further addition of 50 kg ha^{-1} as ammonium nitrate.

On May 25 2011, 15 plants per plot were collected, washed with compressed-air to remove any soil particle, and the fresh weight of both shoot and tap root measured. Twelve roots per plot were used for the root degradation experiment and stored at 4 °C before starting the trial, and 3 roots dried (105 °C, 24 h) for measuring

the moisture content and estimating the total root biomass production on a hectare basis. The dry weight of each of the 12 roots to be buried was used as reference value in the degradation experiment.

Metal analysis and determination of fiber content were performed on dried roots after milling. Metal concentration was measured by ICP-OES after digestion in concentrated HNO_3 according to the USEPA 1995b method and used as reference value for the degradation experiment. The content of acid detergent fiber (ADF), acid detergent lignin (ADL), cellulose and ash (AIA) were measured according to Van Soest (1987).

2.2.2. Root degradation set up

The degradation trial was set up in large boxes filled with either unpolluted or artificially polluted soil. The soil, having a silty-loam texture, was collected on May 2010 at the experimental farm and air-dried in greenhouse for a week. Part of this soil was contaminated by adding Cd, Co Cu and Zn (as sulphate) at a rate of 4, 40, 200 and 750 mg kg^{-1}, respectively using 25 L of contaminated solution mixed with 150 Kg of soil. The contamination level achieved exceeded about 2 times for Cd and Co, and about 5 times for Cu and 8 times for Zn the Italian Guideline Values (IGV) for agricultural soil (Italian Ministerial Decree 252/2006). The soil was then let dry and during the following week it was repeatedly stirred to ensure complete mixing with the metal solution. The reference unpolluted soil was arranged in the same way but without adding the metal solution.

The total metal content in both polluted (P) and unpolluted (UP) soil was measured (USEPA 1995a) to verify the achievement of target concentrations.

The degradation experiment was set up in the open starting on June 7 2011. Each root was wrapped into a 1.2-mm mesh size nylon net bags to avoid soil macrofauna to enter, and placed at 10 cm of depth in PVC boxes (60×40 large, 34 cm height) filled-in with either the polluted or the unpolluted soil. Each root was marked with a small stake and a tag to be recognized at excavation. The boxes were covered with a 1.5-cm mesh size metallic net to avoid any external interference by little animals. The number of roots in each box varied according with the same cultivation

densities adopted during field cultivation, i.e. 44 and 63 plants m^{-2}. During the following 12 months, the roots were periodically collected from the soil (Table 1) for measuring the residual biomass and metal concentrations.

At each collection, the nylon net bags were gently and thoroughly washed with distilled water within PVC boxes to remove the residual soil. Root fragments were collected on a 2 mm mesh sieve. The roots were then dried at 105 °C for 24 h and the dry weight and metal contents were measured as above.

Table 1. Dates of root sampling during the degradation experiment.

Date	Sampling	Days after burying
7/06/2011	Root burying	0
12/07/2011	Sampling 1	35
10/10/2011	Sampling 2	125
9/01/2012	Sampling 3	216
18/04/2012	Sampling 4	316
3/07/2012	Sampling 5	392

2.2.3. Soil analysis

During the degradation experiment, the microbial activity, which might be related to the rate root degradation and metal release, was periodically analyzed. Measurements were carried out through the FDA (Fluorescein diacetate) hydrolysis according to Adam and Duncan (2001). Briefly, the method consisted in placing 2 g of fresh soil in a 50-mL flask with 15 ml of a 60 mM phosphate buffer solution (pH = 7.6, 24 °C). Then 0.2 ml of a FDA stock solution (1000 µg FDA mL^{-1}) was added to start the reaction. Blanks were prepared without adding the FDA substrate, along with a suitable number of sample replicates. The flasks were shaken by hand and placed in an orbital incubator (100 rpm min^{-1}, 30 °C) for 20 min and then added with 15 ml 2:1 (v/v) chloroform-methanol solvent to stop the reaction. After a thorough hand shaking, the samples were centrifuged within 50 ml centrifuge tubes at 2000 rpm min^{-1} for 3 minutes. The supernatant was filtered with Whatman 42 paper filters

for subsequent spectrophotometer absorbance measurement (490 nm wavelength). The concentration of fluorescein released during the reaction was calculated through a calibration curve within the range 0-5 mg fluorescein mL^{-1}, which were prepared from a 20 µg fluorescein mL^{-1} standard solution. The 0 mg ml^{-1} fluorescein standard was used to calibrate the spectrophotometer zero before each set of blanks and samples were read.

Information about the prevailing type of soil microbial activity (cellulolytic and proteolytic) was obtained through the method proposed by Squartini *et al*. (2012). Cotton (three stars, n. 16) and silk (three stars, Bozzolo reale n. 24) 50-cm long treads were buried at about 10 cm of depth in the boxes containing the degrading roots and left for 7, 14 or 21 days during the whole experiment. After incubation, the threads were removed with caution from the soil and their traction resistance was measured through a digital dynamometer (IMADA ZP, Elis, Electronic Instruments and Systems, Roma) with the "peak function" which measures and records the maximum strength (kg) before the tread breaks. The percentage difference between the strength of buried threads and the mean value of the unburied ones was standardized on the number of incubation days in the soil. The percentage variation of the standardized strengths for the cotton and silk threads were assumed as representative of the cellulolytic and proteolytic activities, respectively.

Within boxes, soil bioavailability of Cd, Cu, Ni, Pb and Zn was measured according to Lindsay-Norvell (1978).

Since a spontaneous vegetation flora colonized the soil boxes during the experiment, the abundance of each species was assessed and plant samples collected on October 28 2011, After accurate washing with distilled water, shoot and root samples were oven-dried at 105 °C for 24 h and analyzed by ICP-OES for determining metal concentration as described above.

2.2.4. Climate

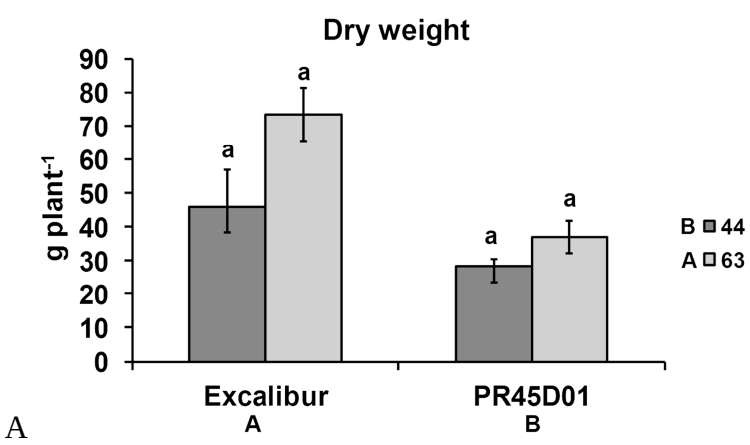
The temperature and humidity contents (Volumetric Water Content) at 10 cm depth in the boxes containing the roots during the degradation experiment was recorded through a data-logger over the whole period of the degradation experiment.

2.2.5. Statistical analysis

All the analysis were performed at least in triplicate. After checking for normality (Skeweness and Kurtosis tests, $P<0.05$) and homogeneity of variances (Bartlett's test, $P<0.05$), the data were analyzed through either ANOVA or Kruskall Wallis test (Costat 6.4, Copyright 1998-2008 CoHort Software 798 Lighthouse Ave. PMB 320 Monterey, CA, 93940, USA). The trend of root degradation and metal release were expressed as residual biomass (percentage of the initial dry weight) and residual metal content, respectively, and interpolated over time (number of days after burying) through the Curve-Expert Professional 1.6.3 software (Copyright 2012, Daniel G. Hyams) and running the analysis CurveFinder for the best fit.

2.3. Results

2.3.1. Shoot and root biomass in rapeseed



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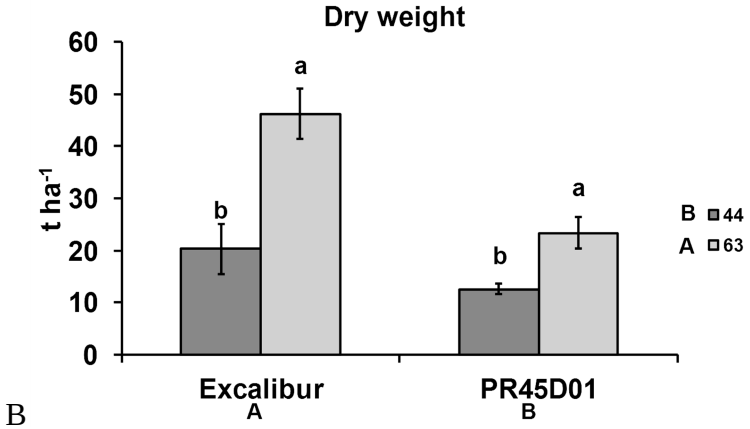

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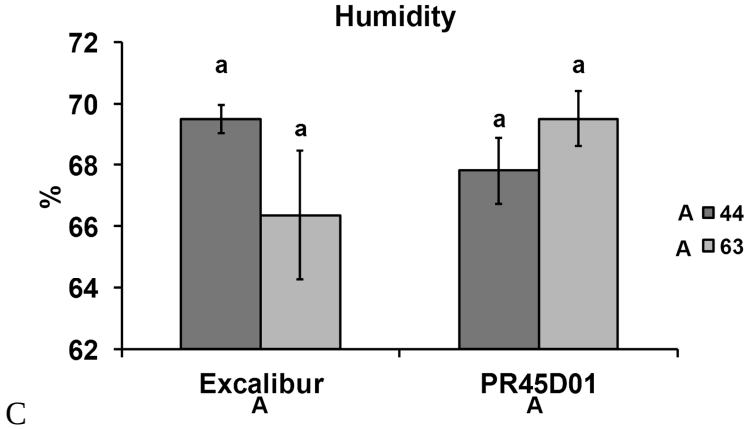


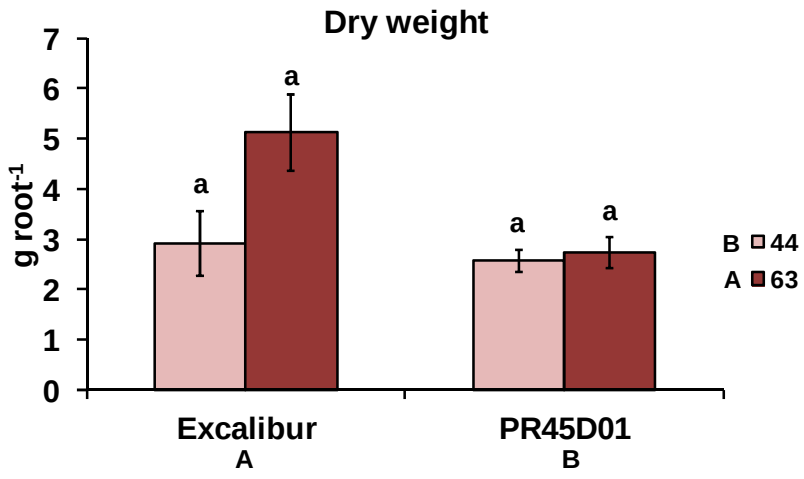
C

Figure 1. Shoot biomass of two rapeseed varieties at two plant densities, approximately at maturity: dry weight per plant (A) and per hectare (B), and % water content (C). Vertical bars represent standard errors. Different letters indicate statistically significant differences (capital letters for main effects; lower case letters between densities within the same cultivar) (Tukey HSD test, $P \leq 0.05$).

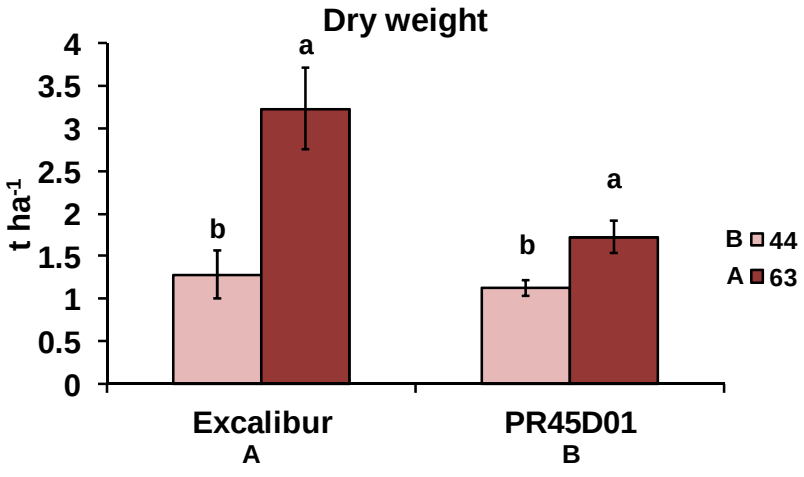
Shoot biomass per plant and per hectare was higher in Excalibur than in the semidwarf hybrid PR45D01 (Fig. 1A, B,) (main effect). The main effect "density" was also significant, with higher biomass for both per plant and per unit surface area at 63 plants m^{-2} (55.1 ± 7.0 g $plant^{-1}$, corresponding to 34.7 ± 4.4 t ha^{-1} vs. 37.1 ± 5.8 g $plant^{-1}$, corresponding 16.3 ± 2.6 t ha^{-1}).

The interaction "cultivar × density" was significant only for the overall per-hectare biomass (Fig. 1B), with the biomass increasing with increasing density for both cultivars. Overall, the highest biomass was produced by Excalibur at 63 plant m^{-2} (73.2 ± 7.8 g $plant^{-1}$, corresponding to 46.2 ± 4.9 t ha^{-1}), and the lowest by PR45D01 at 44 plant m^{-2} (28.3 ± 2.1 g $plant^{-1}$ corresponding to 12.4 ± 0.94 t ha^{-1}).

The water content in shoot tissues was very similar among treatments, irrespective of cultivar, density and their interaction (mean value: 68.3%).



A



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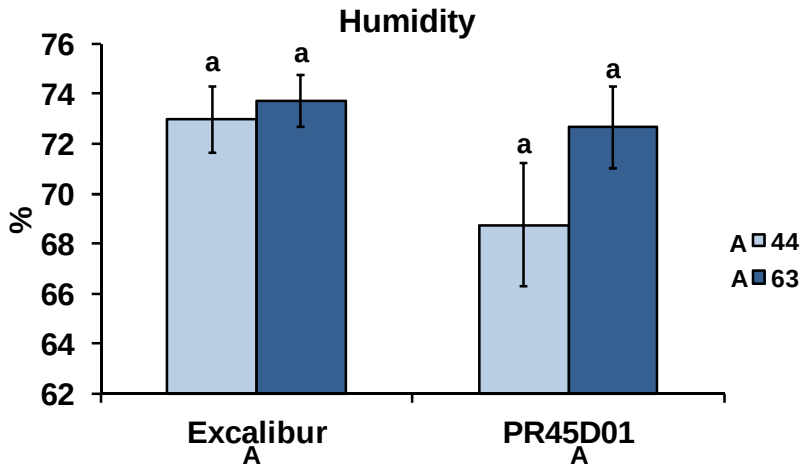

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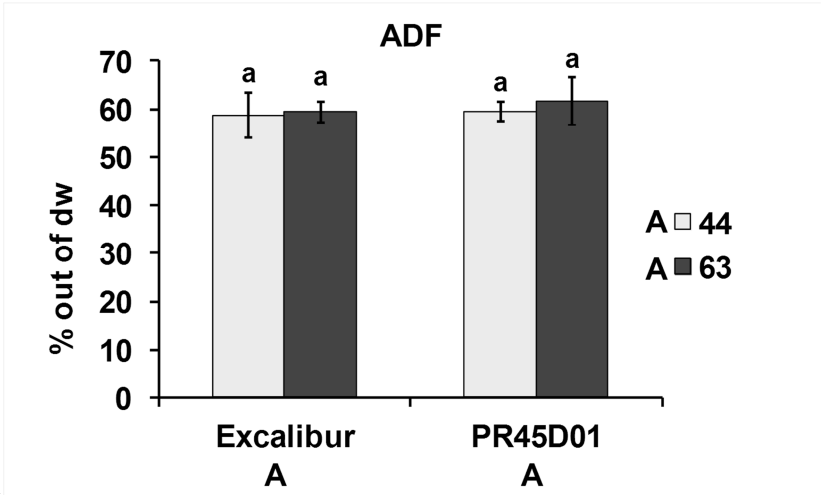
Figure 2. Taproot biomass of two rapeseed varieties at two plant densities, approximately at maturity: dry weight per plant (A) and per hectare (B), and % water content (C). Vertical bars represent the standard errors and different letters indicate statistically significant differences (capital letters for main effects; lower case letters between densities within same cultivar) (Tukey HSD test, *P*≤0.05).

At root level, dry biomass per plant was significantly higher in Excalibur (4.02 ± 0.56 g dw) than in PR45D01 (2.7 ± 0.18 g dw) (Fig. 2A). Consequently, the total root biomass produced per hectare was significantly higher in Excalibur (2.2 ± 0.39 t ha^{-1}) than in PR45D01 (1.4 ± 0.13 t ha^{-1}) (Fig. 2B).

Root biomass was always significantly higher at the highest plant density, but the interaction "cultivar × density" was significant only for the "per-hectare biomass". The highest root biomass was produced by Excalibur at 63 plant m^{-2} (5.1 ± 0.77 g $root^{-1}$, corresponding to 3.2 ± 0.48 t ha^{-1}) and the lowest biomass by PR45D01 at 44 plant m^{-2} (2.6 ± 0.22 g $root^{-1}$, corresponding to 1.3 ± 0.09 t ha^{-1}). The incidence of taproot biomass on total plant weight ranged between 9.5% to 6.5 %.

The percentage of humidity in root tissues was very stable, irrespective of cultivar, density and their interaction (mean value: 72%).

2.3.2. Fiber Content in Taproots



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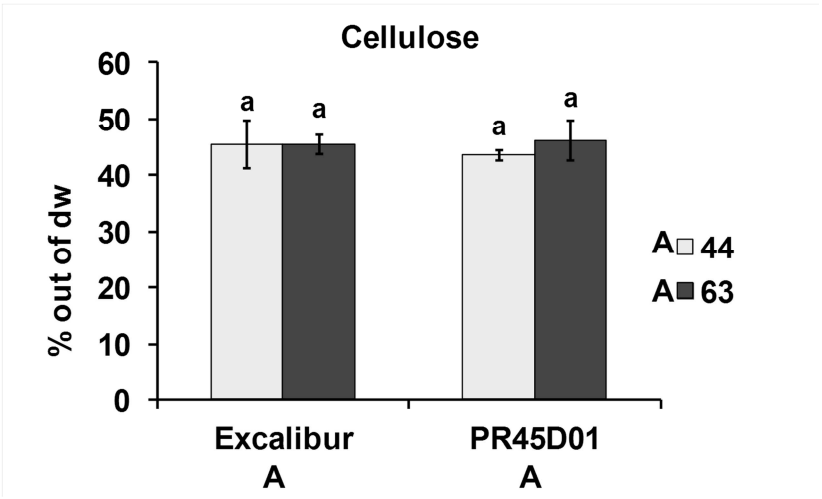

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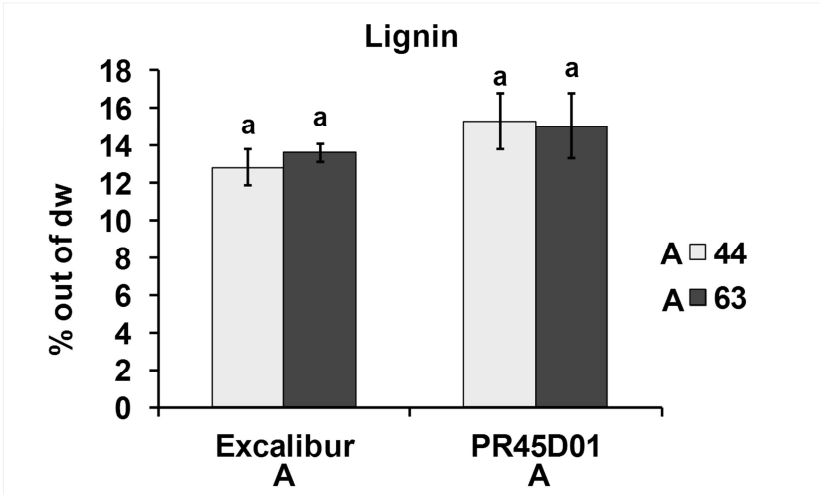

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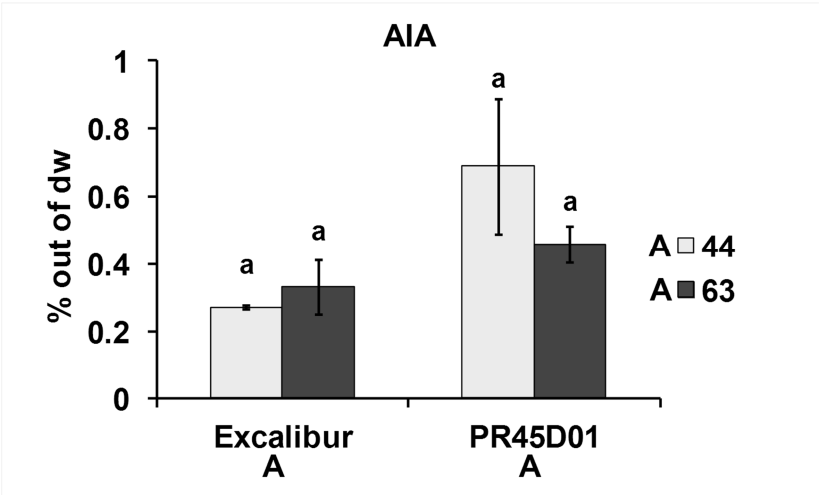


D

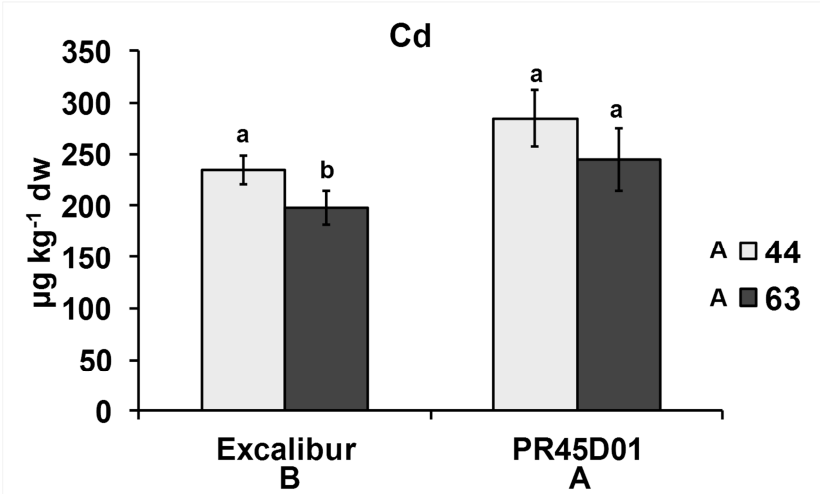
Figure 3. Fiber contents in taproots approximately at plant maturity (% out of dw). Vertical bars represent standard error. Different letters indicate statistically significant differences (capital letters for main effects; lower case letters between densities within same cultivar) (Tukey HSD test, *P*≤0.05).

The content of fiber (ADF), cellulose, lignin and ash (AIA) in the taproots at harvest was the same irrespective of genotype and plant density. The average values were 59 ± 5.68% dw, 45 ± 4.46%, 14 ± 2.1% and 0.43 ± 0.23% for ADF, cellulose, lignin and ash, respectively. At the end of the root degradation experiment, the residual fiber content was lower than at the beginning, but no differences were found between soil treatments, genotypes or plant densities. Among parameters, only lignin and AIA increased over time. At the end of the experiment, about one year later, the average values in the polluted soil were 55 ± 3.3%, 30 ± 4.4%, 23 ± 1.4% and 2.2 ± 0.58%, for ADF, cellulose, lignin and ash, respectively, whereas those of the unpolluted soil were 54 ± 3.6%, 28 ± 4.4%, 25 ± 0.87% and 1.9 ± 0.24%, respectively.

2.3.3. Heavy Metals

Metal concentration and removals in shoots

A



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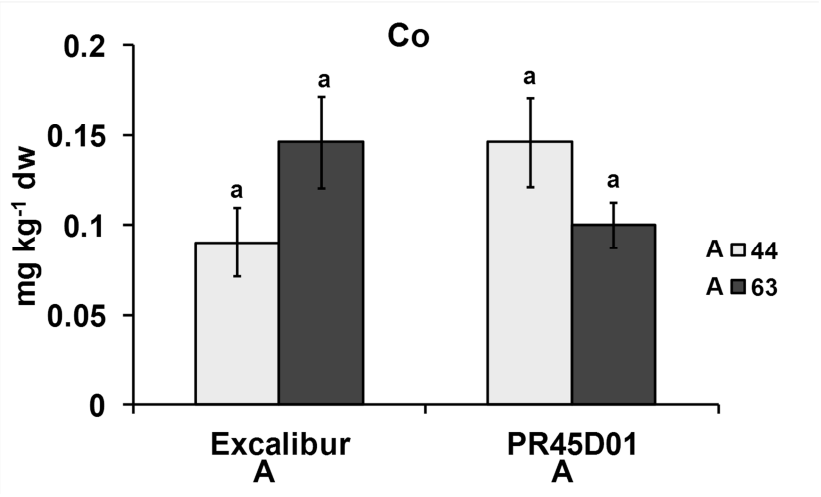

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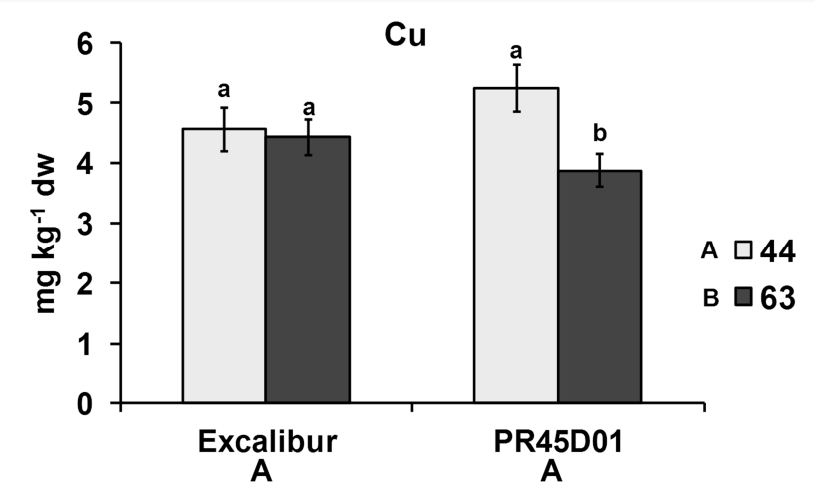

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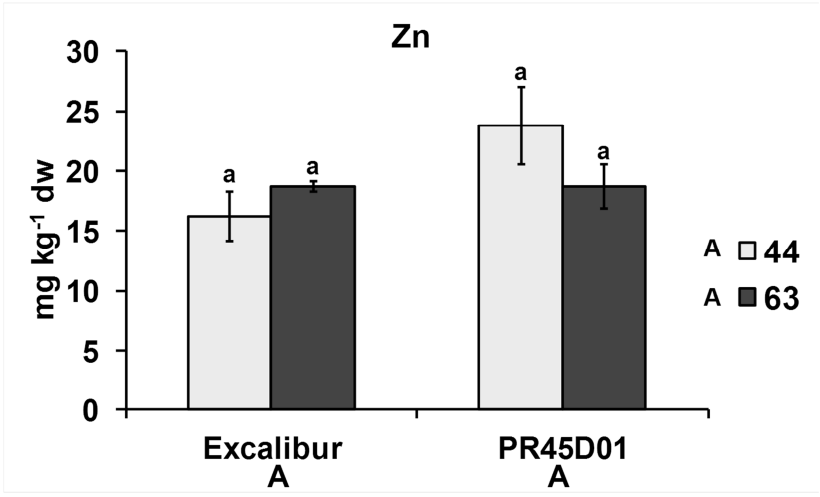
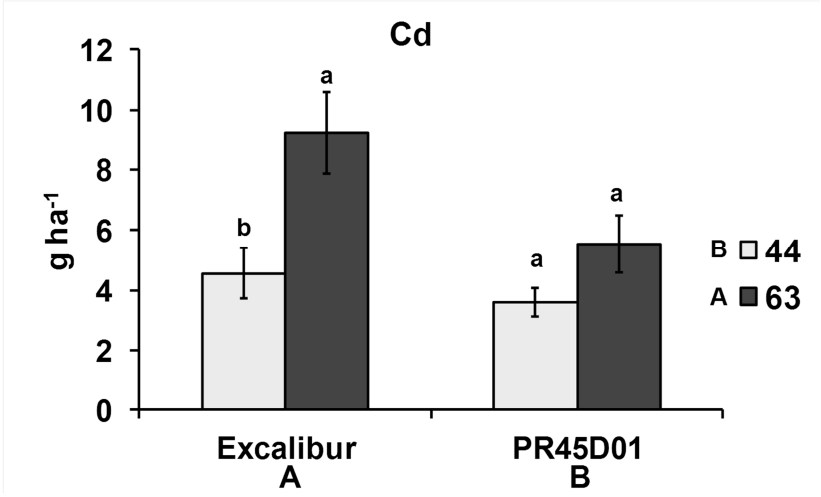


D

Figure 4. Metal concentrations (mg kg^{-1} dw) in shoots at harvest of rapeseed varieties under two plant densities. Different letters indicate statistically significant differences (capital leters for main effects; lower case letters between densities within same cultivar) (Tukey HSD test, *P*≤0.05;). Vertical bars represent standard error.

Metal concentration in shoots was relatively constant among treatments, irrespective of cultivar or density, except for Cd, the semidwarf hybrid PR45D01 having significantly higher values (264 ± 21 µg kg^{-1} dw vs. 216 ± 12 µg kg^{-1} dw of Excalibur), and for Cu the plant density of 44 plant m^{-2} leading to significantly higher concentrations (4.90 ± 0.27 mg kg^{-1} vs. 4.14 ± 0.21 mg kg^{-1} of the 63 plants m^{-2}).

The interaction "cultivar × density" was significant only for Cd in Excalibur (Fig. 4A) and Cu in PR45D01 (Fig.4C), with the highest plant density having the lowest concentration.



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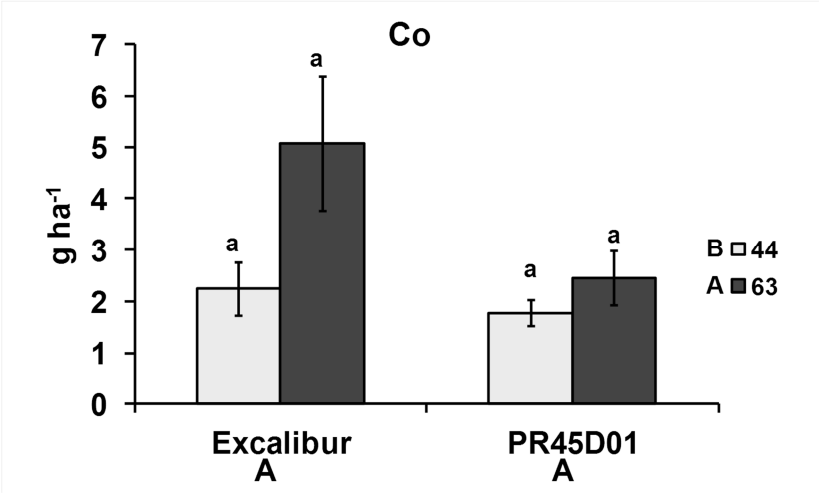

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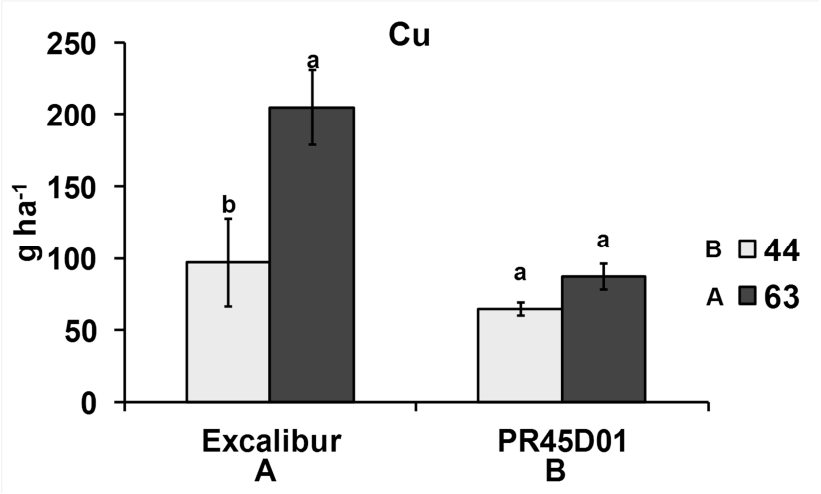

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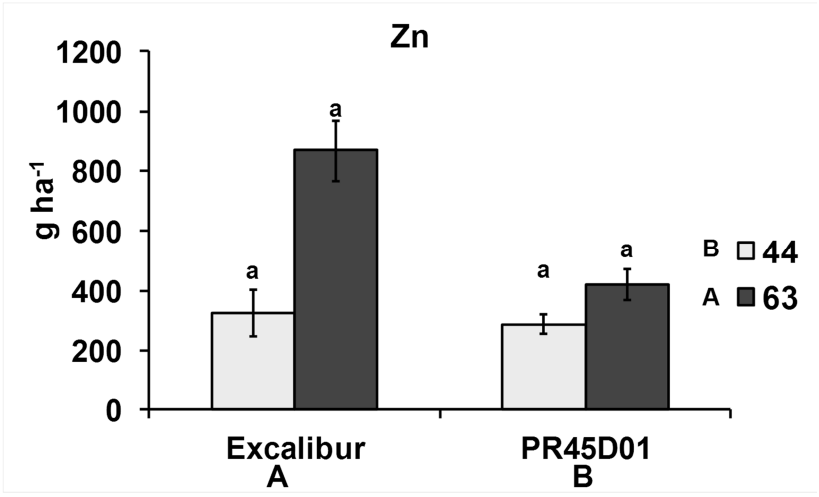

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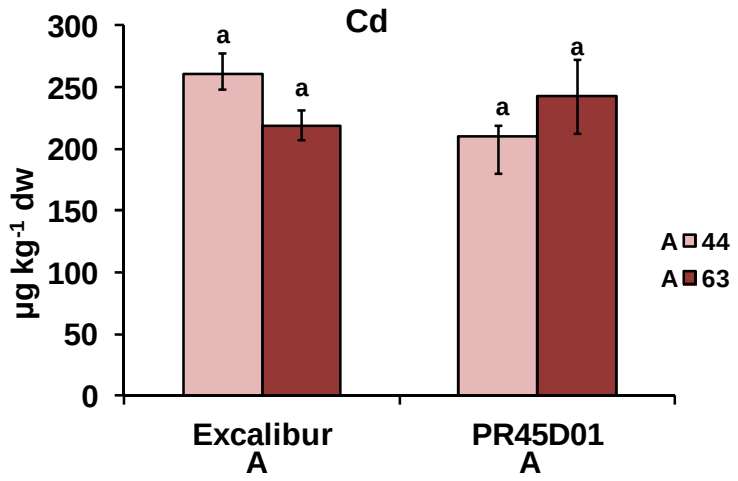
Figure 5. Metal removal (g ha^{-1}) by the above-ground biomass of rapeseed varieties at two plant densities at harvest. Different letters indicate statistically significant differences (capital letters for main effects; lower case letters between densities within same cultivar) (Tukey HSD test, *P*≤0.05). Vertical bars represent standard error.

The content of heavy metals in shoots was significantly higher in Excalibur than PR45D01 for all the elements, except Co. The main effect "plant density" was also significant for all metals, with removals being significantly higher at 63 plants m^{-2} than 44 plants m^{-2}. These results were related to better productivity achieved with Excalibur and under higher sowing density.

The interaction "cultivar × density" was significant only for Cd and Cu in Excalibur, with removals increasing with plant density.

Metal concentration and removals in taproots



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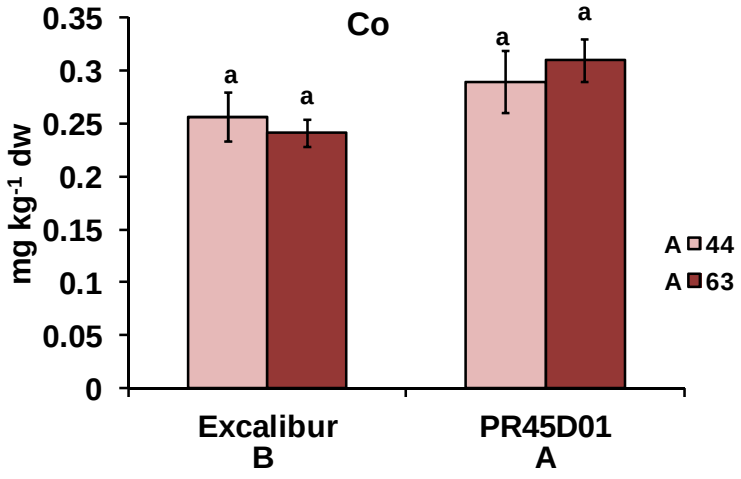

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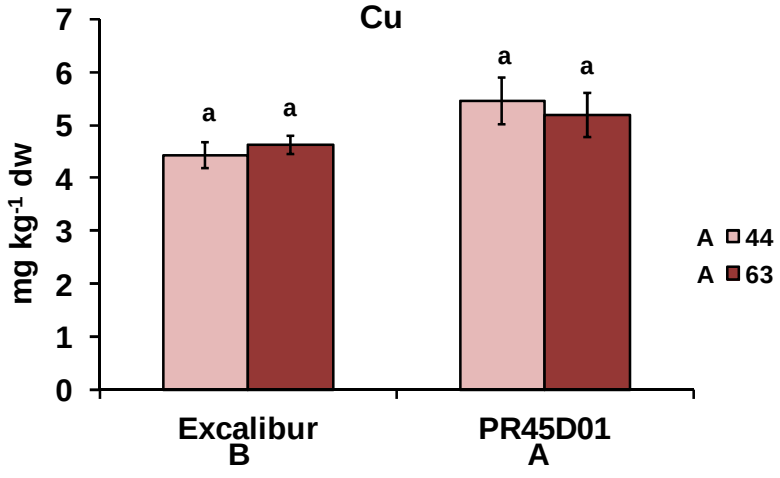

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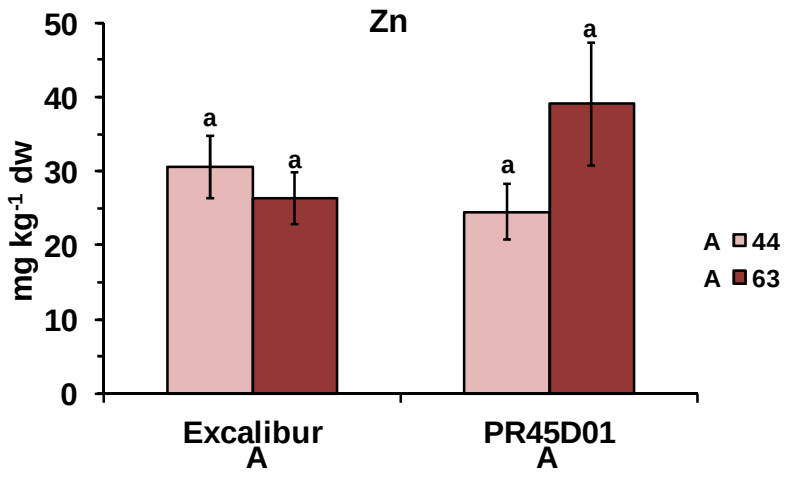

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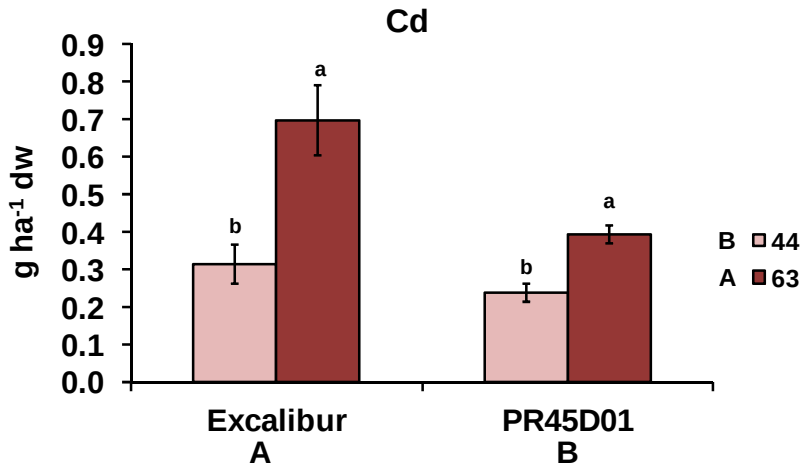
Figure 6. Metal concentrations (mg kg^{-1}) in taproots of rapeseed varieties at two plant densities at harvest. Different letters indicate statistically significant differences (capital letters for main effects; lower case letters between densities within same cultivar) (Tukey HSD test, *P*≤0.05). Vertical bars represent standard error.

Metal concentrations in taproots did not vary greatly among treatments. It was constant irrespective of plant density, whereas significant differences were found between cultivars for Co and Cu, with Excalibur having lower concentrations than PR45D01 (0.25 ± 0.01 and 0.30 ± 0.02 mg Co kg^{-1} dw, and 4.25 ± 0.15 and 5.32 ± 0.29 mg Cu kg^{-1} dw, for Excalibur and PR45D01 respectively). The mean Cd and Zn concentrations were 233 ± 10 µg kg^{-1} dw and 30.1 ± 2.7 mg kg^{-1} dw, respectively.

The interaction "cultivar × density" was not significant for any of the metals considered.



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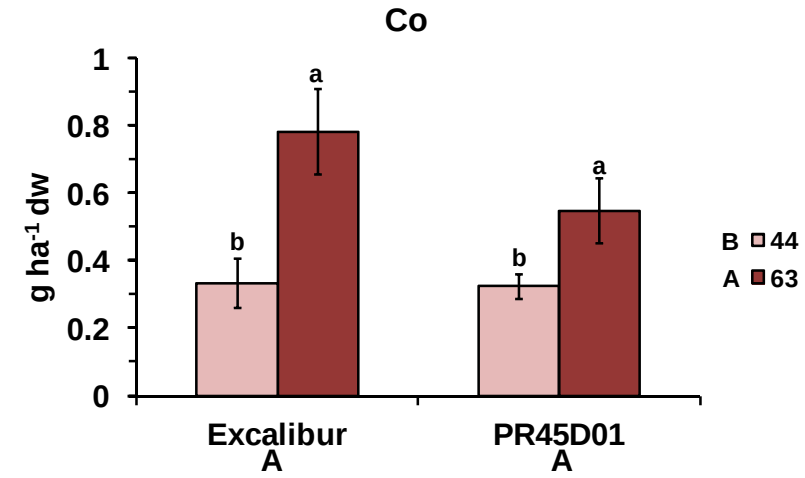

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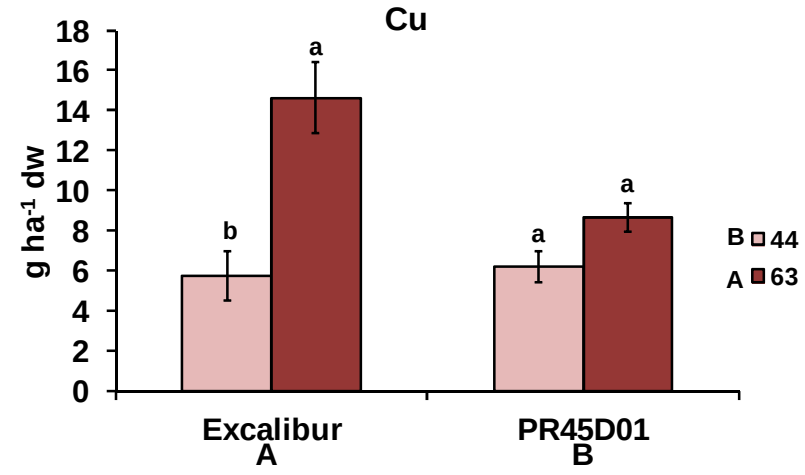

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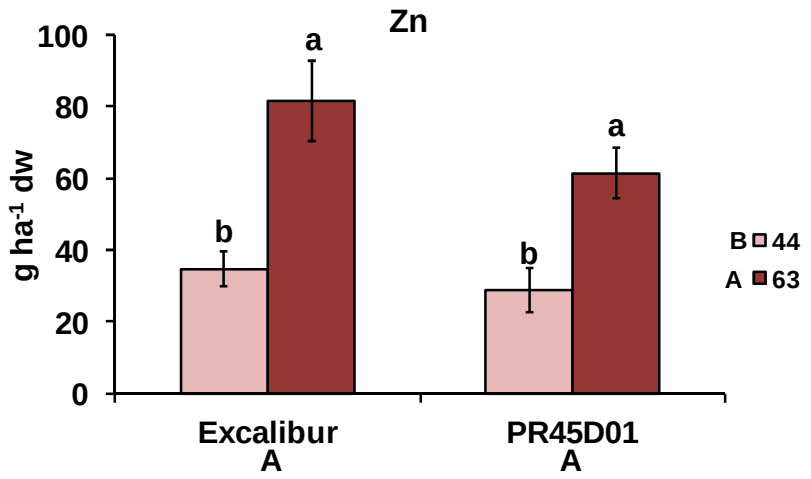

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Figure 7. Metal removals by taproots (g ha^{-1}) of two rapeseed varieties at two plant densities at harvest. Different letters indicate statistically significant differences (capital letters for main effects; lower case letters between densities within same cultivar) (Tukey HSD test, *P*≤0.05). The vertical bars represent the standard error.

Metal removals by taproots was generally higher at increased plant density (i.e., 63 plant m^{-2}). Significant differences were also found for Cd and Cu between cultivars, with Excalibur having higher contents of both metals (0.51 ± 0.0006 vs. 0.32 ± 0.01 g Cd ha^{-1} of Excalibur and PR45D01 respectively, and 10.1 ± 0.2 and 7.4 ± 0.2 g Cu ha^{-1} of Excalibur and PR45D01 respectively).

The interaction "cultivar × density" was always significant and metal removals improved with increasing plant density, except for Cu in PR45D01.

2.3.4. Dynamics of taproot degradation

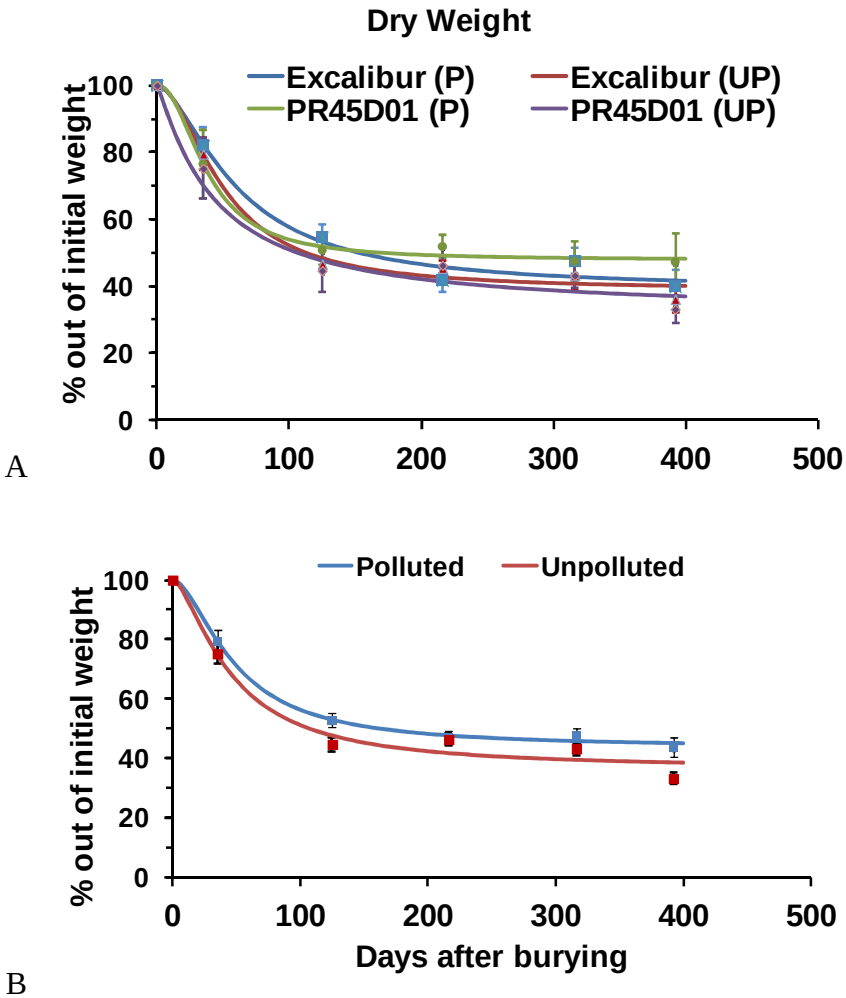


Figure 8. Residual dry weight of degrading taproots (% out of the initial dry weight) as measured during the experiment (squares, circles, triangles) and predicted trends (lines). Interaction "cultivar × soil" (A): Excalibur in polluted (Excalibur P) and unpolluted soil (Excalibur UP) as blue squares and line, and red triangle and line, respectively; PR45D01 in polluted (PR45D01 P) and unpolluted soil (PR45D01 UP) as green circle and line, and violet squares and line, respectively. Main effect "soil contamination" (B): polluted (blue squares and line) and unpolluted (red squares and line). Vertical bars represent standard error. The model used was a MMF model [$y = (a \times b + c \times x^d)/(b + x^d)$].

At all sampling dates, no statistically significant differences were found in the residual weight for the main effects cultivar, plant density or their interaction, nor for the interaction "cultivar × soil", "plant density × soil" and "cultivar × plant density × soil". Nevertheless, since the main effect "soil contamination" resulted significant for the % residual dry weight (% residual dw), here it is reported only the results for the

interaction “cultivar × soil” (Fig. 8A) and the main effect “soil contamination” (Fig. 8B).

PR45D01 had produced a significantly smaller root biomass (1.4 ± 0.13 t ha^{-1}) than Excalibur (2.2 ± 0.39 t ha^{-1}), but in the polluted soil at the end of the experiment PR45D01 maintained a higher biomass (mean: 1.4 ± 0.11 g per root, corresponding to 713 ± 68 kg ha^{-1}) than Excalibur (1.1 ± 0.15 g per root, corresponding to 580 ± 91 kg ha^{-1}), although the difference was not statistically significant. The corresponding percentages of residual biomass were 47 ± 5.5 % dw and 40 ± 3.5 % dw for PR45D01 and Excalibur respectively. In the unpolluted soil, at the end of the experiment the highest residual biomass was found in Excalibur (1.8 ± 0.40 g per root, corresponding to 920 ± 162 kg ha^{-1}), whereas PR45D01 resulted in a lower residual biomass (1.3 ± 0.24 g per root, corresponding to 710 ± 108 kg ha^{-1}), but the difference was not statistically significant. The corresponding percentages of residual biomass were 36 ± 2.7 % dw and 30 ± 2.6 % dw for Excalibur and PR45D01 respectively. Overall, irrespective of plant density and soil contamination, Excalibur and PR45D01 preserved the same residual root biomass (38 ± 2.2%), for both per plant (1.4 ± 0.12 g per root) and per hectare base (731 ± 58 kg ha^{-1}).

The degradation was faster in the unpolluted soil, since the % of residual biomass (33 ± 2.0 % dw) was significantly lower than in the polluted soil (43 ± 3.3% dw).

The % residual weight for the interaction “cultivar × soil” and the main effect “soil” was approximated through the MMF model

$$y = (a * b + c * x^d)(b + x^d)$$

which belong to the family of the sigmoidal functions.

Table 2. Coefficient of the MMF model and coefficient of determination (R^2) for the equation describing the % of residual taproot dry weight for the interaction "cultivar × soil contamination" and the main effect "soil".

Coefficient	Polluted soil			Unpolluted soil		
	Excalibur	PR45D01	Average	Excalibur	PR45D01	Average
a	99.94	100.01	100.00	100.08	100.11	100.10
b	806.06	2217.82	724.93	1406.96	72.33	351.93
c	39.03	47.79	43.33	38.65	31.76	36.36
d	1.630	2.112	1.698	1.850	1.136	1.532
R^2	0.985	0.996	0.998	0.990	0.960	0.977

The goodness of the degradation trends was assessed by regressing the values of the residual root biomass measured during the experiment and the corresponding values found through the equation for each interaction "cultivar × soil" and the main effect "soil". Cross validation indicated that there was always a good correlation between measured and predicted data (Table 3).

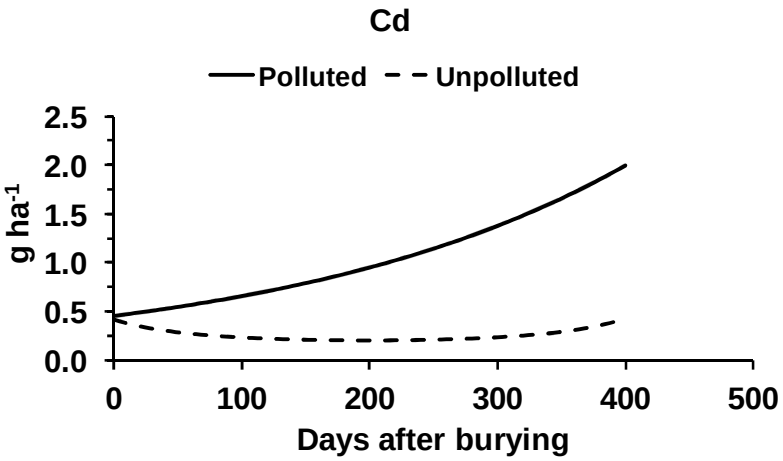
Table 3. Statistics of linear regression between measured (x) and simulated (y) data values of % residual taproot biomass. CL indicates the 95% confidence limit. When "*P*" is lower than 0.05, the coefficient (slope and intercept) is significantly different from zero.

Parameter	Polluted			Unpolluted		
	Excalibur	PR45D01	Average	Excalibur	PR45D01	Average
Slope ± CL	0.981 ± 0.166	0.991 ± 0.094	0.993 ± 0.0684	0.985 ± 0.3001	0.957 ± 0.275	0.974 ± 0.209
P value (slope)	0.0001***	0.0000***	0.0000***	0.0021**	0.0006***	0.0002***
Intercept ± CL	1.097 ± 10.792	0.470 ± 6.16	0.342 ± 4.45	0.79 ± 15.7	2.34 ± 16.6	1.46 ± 12.9
P value (intercept)	0.792 (ns)	0.843 (ns)	0.841 (ns)	0.660 (ns)	0.715 (ns)	0.769 (ns)

The estimated half-time for biomass loss/degradation ($t_{(1/2)}$) was 154 and 114 days for Excalibur in the polluted and unpolluted soil respectively, and 167 and 105 days for PR45D01 in the polluted and unpolluted soil respectively.

According to the model, the loss of dry weight appears faster in the unpolluted soil over the whole period of degradation (Fig. 8B) and $t_{(1/2)}$ was 159 and 107 days in the polluted and unpolluted soil, respectively.

2.3.5. Residual metal contents in taproots during degradation



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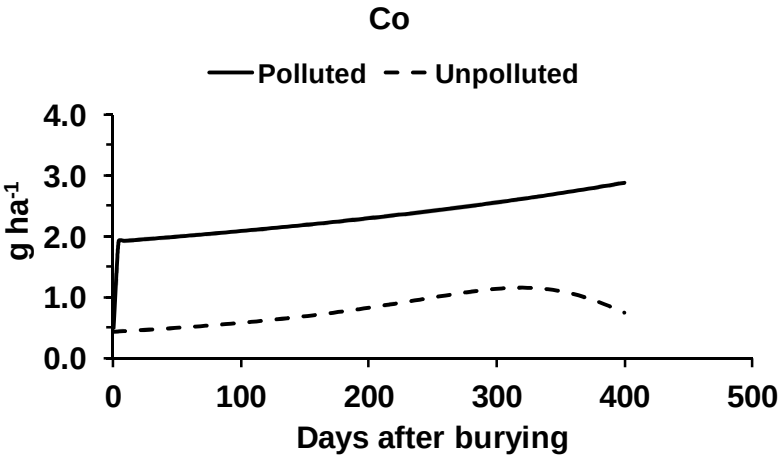

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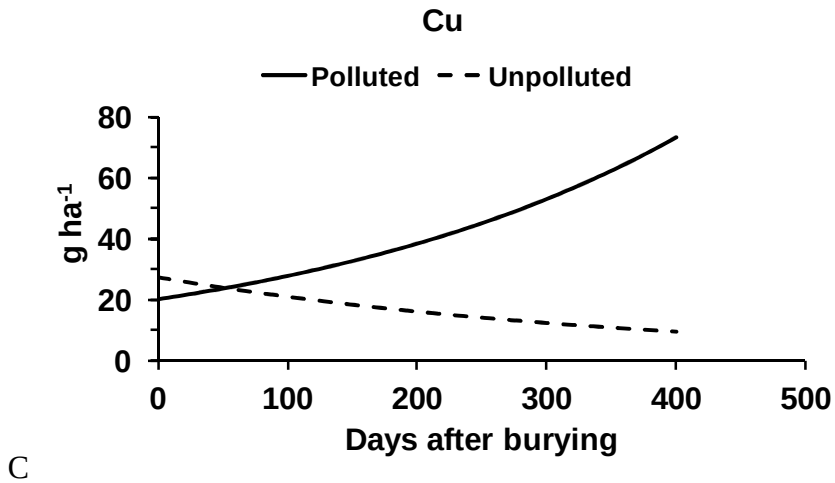

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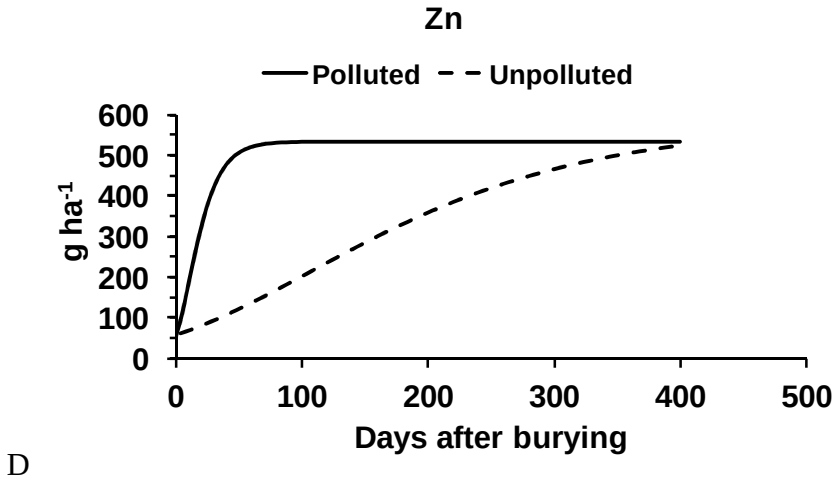

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Figure 9. Estimated trends of residual metal contents in taproots of rapeseed for the main effect "soil contamination".

Table 4. Model, equation and coefficient for the functions estimated to predict the residual metal content (g ha^{-1}) in roots during degradation.

Metal	Model	Equation	Coefficient				
			a	b	c	d	R^2
	Polluted Soil						
Cd	Exponential	$y = ae^{bx}$	4.50E-01	3.73E-03			0.83
Co	Rational	$y = (a+bx)/(1 + cx + dx^2)$	4.96E-01	6.88E+05	3.61E+05	-3.03E+02	0.68
Cu	Exponential	$y = ae^{bx}$	2.00E+01	3.25E-03			0.79
Zn	Gompertz	$y = ae^{-e^{\wedge}(b-cx)}$	5.34E+02	8.46E-01	7.54E-02		0.76
	Unpolluted Soil						
Cd	Reciprocal Quadratic	$y = 1/(a + bx + cx^2)$	2.44E+00	2.41E-02	-6.07E-05		0.91
Co	Rational	$y = (a+bx)/(1 + cx + dx^2)$	4.22E-01	-8.50E-04	-4.69E-03	6.17E-06	0.93
Cu	Exponential	$y = ae^{bx}$	2.71E+01	-2.69E-03			0.15
Zn	Gompertz	y = ae-e^(b-cx)	5.78E+02	8.14E-01	7.91E-03		0.89

Metal concentrations (mg kg^{-1}) and contents (mg ha^{-1}) in degrading taproots were measured periodically at the same time as the residual biomass. Concentration and total content of metals increased over time, and no statistically significant differences were found for both the main effects “cultivar” and “density” or their interaction.

On the contrary, significant differences were found between the two soils in the content of HM, with roots buried in the polluted soil always having higher values than those buried in the unpolluted one. For this reason, only results for the main effect “soil contamination” are reported here.

The trends for the residual metal contents are shown in Fig. 9. For each metal, the data were interpolated with the same model, irrespective of soil contamination level (Rational Model for Co, Exponential for Cu and Gompertz for Zn), except for Cd, the function of which was Exponential in polluted soil and Reciprocal Quadratic in the unpolluted one.

The goodness of each function was test through cross-validation and results are reported in Table 5.

Table 5. Statistics for cross-validation between measured (x) and simulated (y) values of residual metal contents in taproots of rapeseed (g ha^{-1}) over time. CL indicates the 95% confidential limit. When *P*<0.05, the coefficient (slope and intercept) is significantly different from zero.

Metal	R^2	Slope ± CL	P value (Slope)	Intercept ± CL	P value (Intercept)
Polluted Soil					
Cd	0.83	0.83 ± 0.16	0.011*	0.17 ± 0.61	0.481 (ns)
Co	0.84	0.42 ± 0.65	0.044*	1.2 ± 1.47	0.283 (ns)
Cu	0.79	0.78 ± 0.55	0.017*	9.0 ± 24.9	0.373 (ns)
Zn	0.76	0.76 ± 0.59	0.024*	107.6 ± 288.2	0.358 (ns)
Unpolluted Soil					
Cd	0.91	0.93 ± 0.53	0.011*	0.020 ± 0.164	0.721 (ns)
Co	0.93	0.96 ± 0.47	0.008*	0.038 ± 0.381	0.807 (ns)
Cu	0.15	0.15 ± 0.50	0.452 (ns)	15.2 ± 12.2	0.026*
Zn	0.89	0.89 ±0.56	0.015*	39.4 ± 213.8	0.599 (ns)

Generally there was a good correlation (high R^2) between measured and estimated values, especially for Cd and Co, irrespective of soil contamination level. The correlation was always significant ($P \leq 0.05$ for slope), except for Cu in the unpolluted soil. For Cu in the unpolluted soil, it was difficult to identify a robust model, and the residual content was therefore described by the same model as that in the polluted soil.

2.3.6. Soil microbial activity

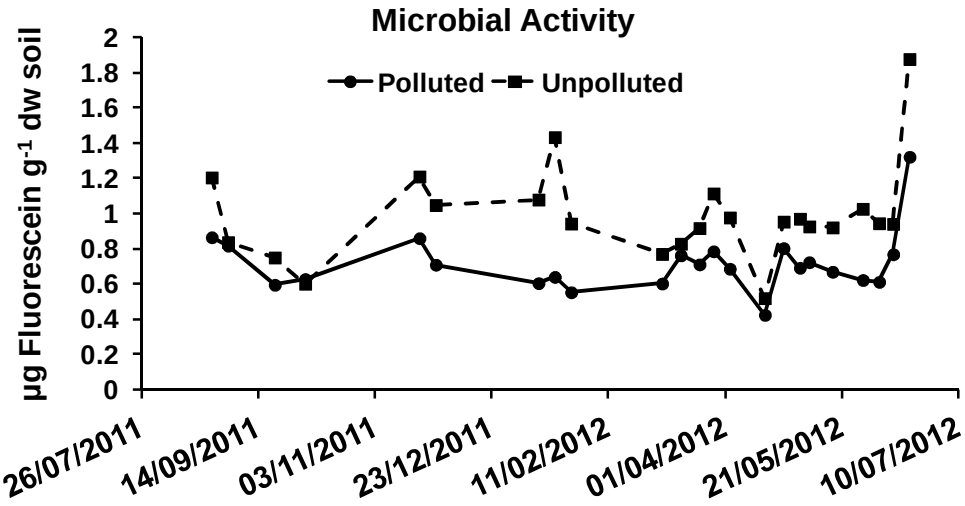


Figure 10. Soil microbial activity measured through the Fluorescein method during the period of root degradation.

The dynamics of soil microbial activity for the main effect "soil contamination" during the experiment is reported in Fig. 10. The activity changed over time in both soils, but generally it was greater in the control unpolluted soil than in the polluted treatment.

The prevailing type of microbial activity is shown in Fig. 11. The "fertimeters" were used over the whole period of root degradation, but in 2011 they were incubated in the soil for 7 days, whereas in 2012 for 14 or 21 days. The original method (Squartini et al, 2012) suggested 7 days of soil incubation, but this was believed to be insufficient to detected differences between the polluted and unpolluted soils. To compare results, the data of % strength were therefore normalized by the time span (days) between burying and collection of the fertimeters from the soil.

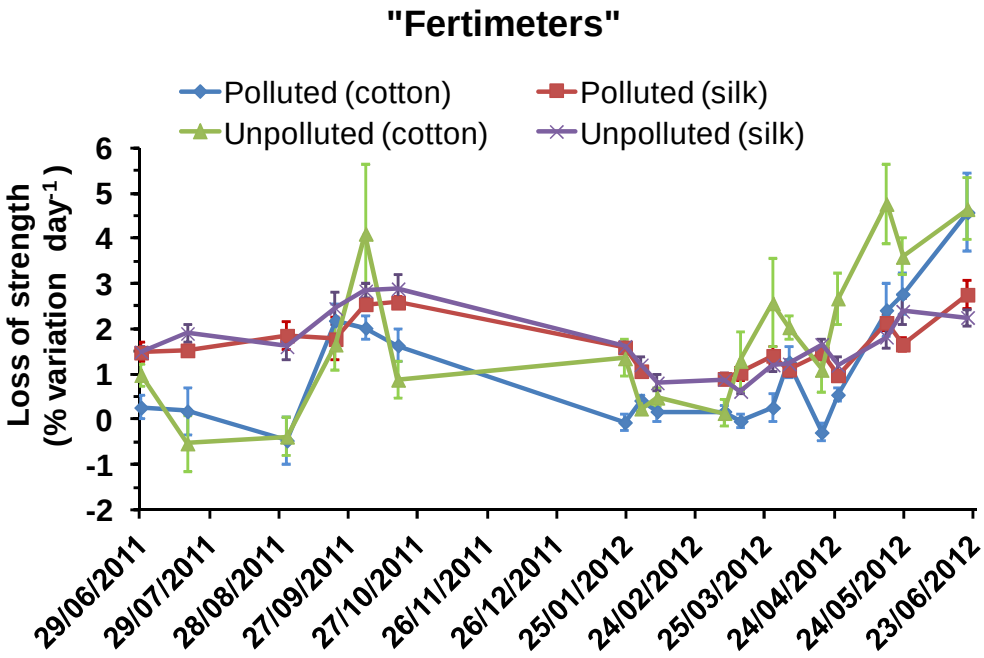


Figure 11. Strength percentage variation of cotton or silk thread (fertimeters) standardized by the incubation time in soil. The curves are representative of the microbial activity type in the two soils. Vertical bars represent standard error.

Cotton thread (cellulose) is generally more recalcitrant to degradation than silk, but at the end of the experiment the cellulolytic activity seemed to prevail over the proteolytic activity, especially in the unpolluted soil. In addition, as a general trend, in the unpolluted soil the degradation of both thread types was greater than in the polluted soil. Nevertheless, the proteolytic activity was more similar between two soils than the cellulolytic one.

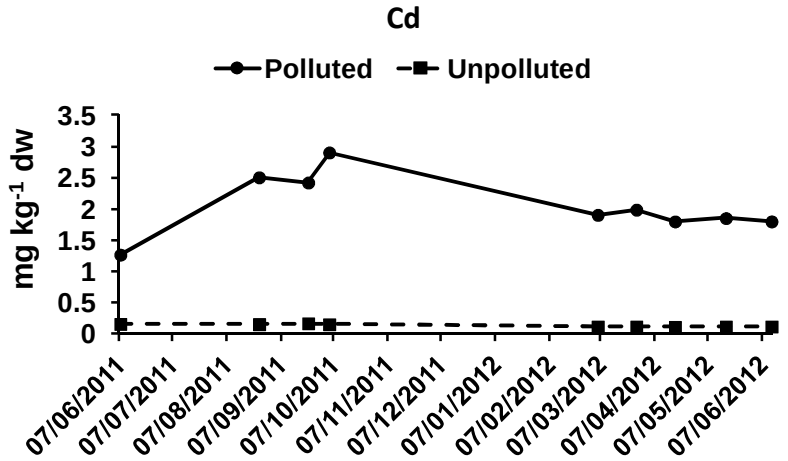
2.3.7. Total and bioavailable heavy metal

Table 6. Pseudo-total metal concentration (mg kg^{-1} dw) in polluted and unpolluted soils before root burying and Italian Guidelines Values (IGV) for agricultural soils (Ministerial Decree 152/2006). Highlighted values (bold) above IGV.

Metal	IGV (mg kg^{-1} dw)	Soil	
		Polluted	Unpolluted
Cd	2	**4.60 ± 0.47**	0.047 ± 0.011
Co	20	**48.5 ± 3.8**	9.95 ± 0.15
Cu	120	**751 ± 92**	36.4 ± 0.5
Ni	-	22.8 ± 0.7	22.0 ± 0.4
Pb	100	22.1 ± 0.8	23.0 ± 0.4
Zn	150	**1278 ± 130**	83.7 ± 1.4

The metals added to the polluted thesis (i.e., Cd, Co, Cu and Zn) reached the expected concentration (Table 6) and exceed the legal limits by about two times (Cd and Co), four times (Cu) and 8 times (Zn).

In the reference unpolluted soil, all elements did not exceed IGV and, as expected, Ni and Pb had the same concentration in both soils (Table 6).



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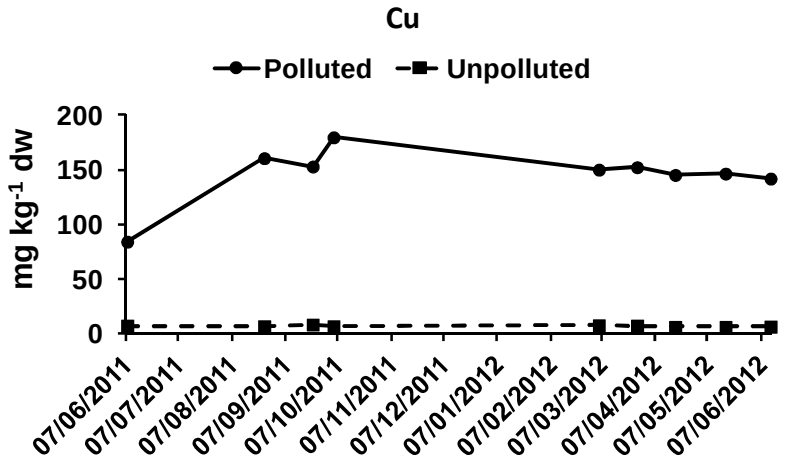

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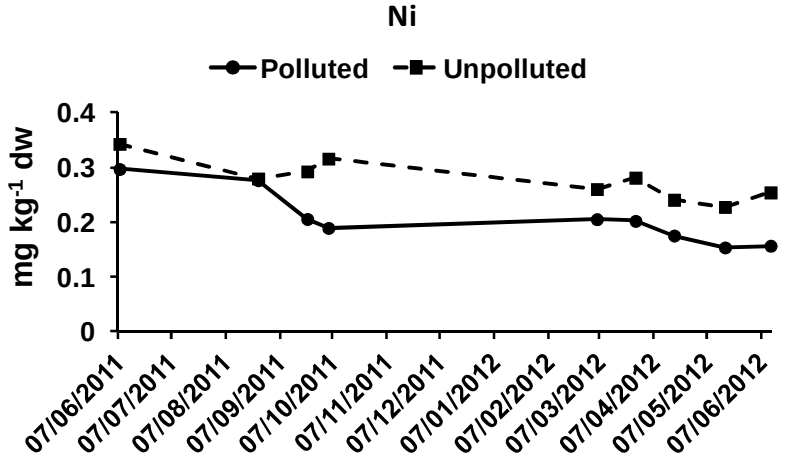

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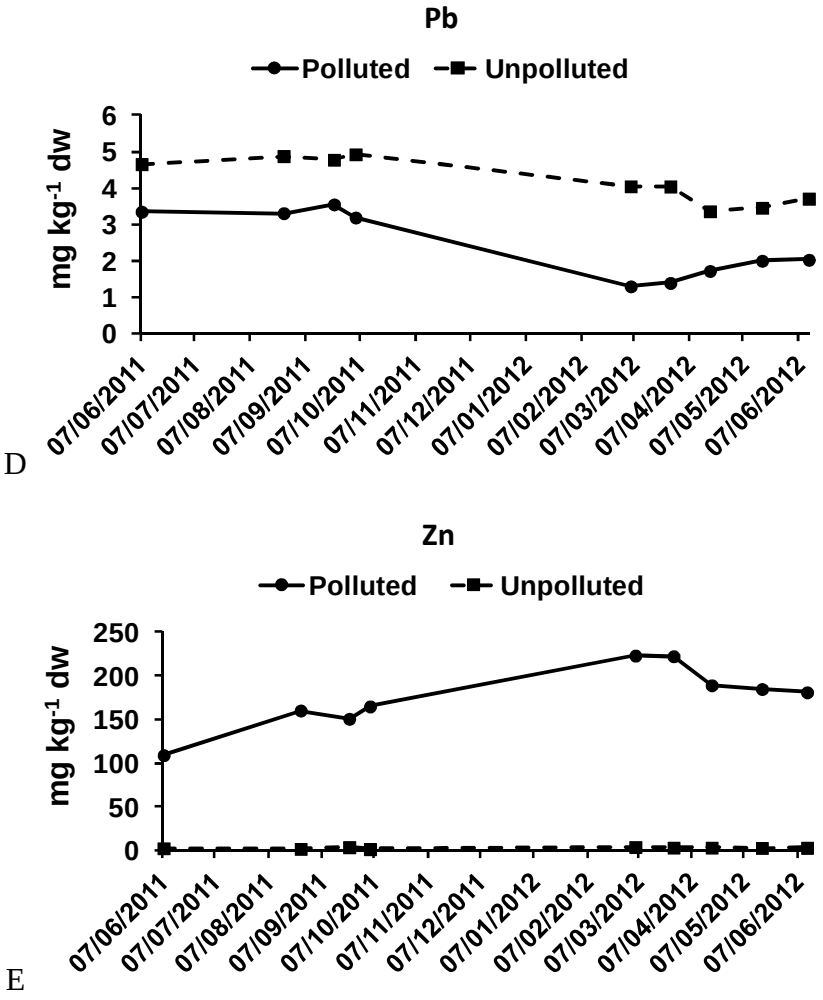


Figure 12. Bioavailability (mg kg^{-1} dw) of metals in polluted and reference unpolluted soil determined according to the Lindsay-Norvell (1979) method. Note the different scale for each metal.

In the polluted soil, the added contaminants (Cd, Cu and Zn) had greater bioavailability than in the unpolluted reference soil. In contrast, Ni and Pb had higher bioavailability in the unpolluted treatment. The bioavailability of Co was not measured because the determination method does not consider this element.

The dynamics of metal bioavailability changed over time in the artificially polluted soil. Cd, Cu and Zn bioavailability rose up during the first part of the experiment (2011), whereas later it tended to decrease and stabilize. For Zn, the bioavailability increased until march 2012, then started to decrease.

In the reference unpolluted thesis, all the metals maintained a relatively stable bioavailability over time.

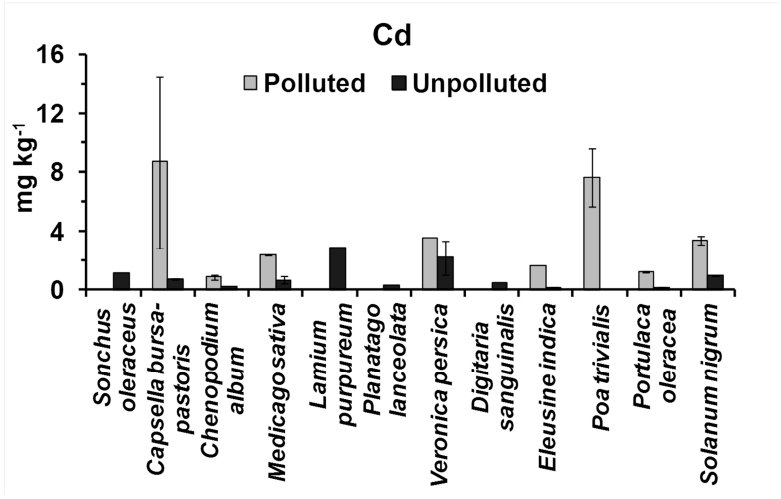
2.3.8. Spontaneous vegetation in polluted soil

The plants spontaneously growing in the experimental boxes were recognized as a typical flora of the North Italy environment. There were some differences in the composition of the community depending on the pollution level (polluted vs. unpolluted soil).

Some species (e.g., *Lamium purpureum* L., *Plantago lanceolata* L.) were found only in the unpolluted thesis; in contrast, *Poa trivialis* L. was found only in the polluted one. However, most species were found in both the polluted and the unpolluted soil.

The most abundant species in both soils were *Capsella bursa-pastoris* L., which was more abundant in the unpolluted soil, and *Portulaca oleracea* L.. Other species were: *Sonchus oleraceus* L., *Chenopodium album* L., *Medicago* sativa L., *Veronica persica* L., *Digitaria sanguinalis* L., *Solanum nigrum* L., *Eleusine indica* (L.) Gaertn.

The concentration of heavy metals in shoot and roots are reported in Fig. 13 and 14 respectively.



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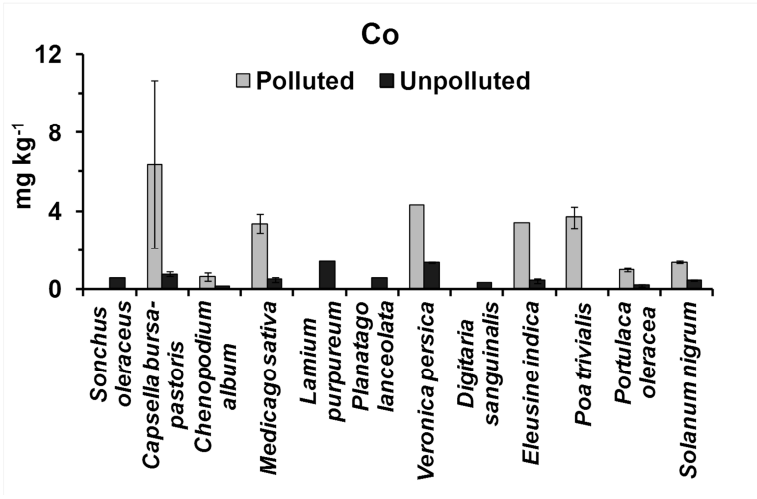

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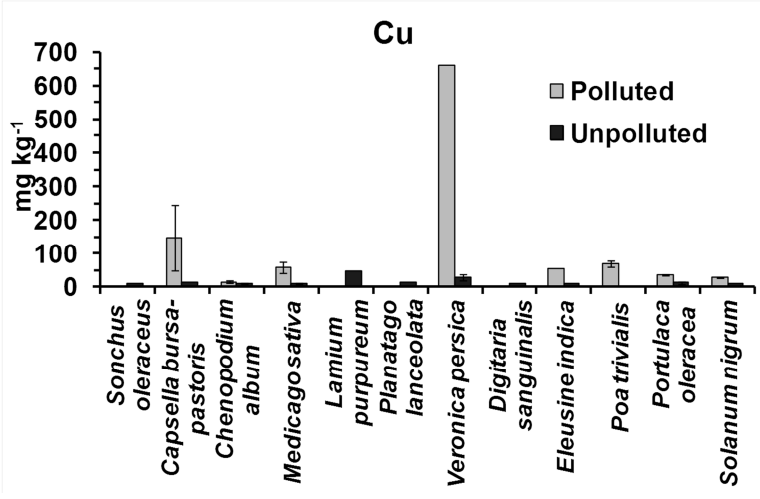

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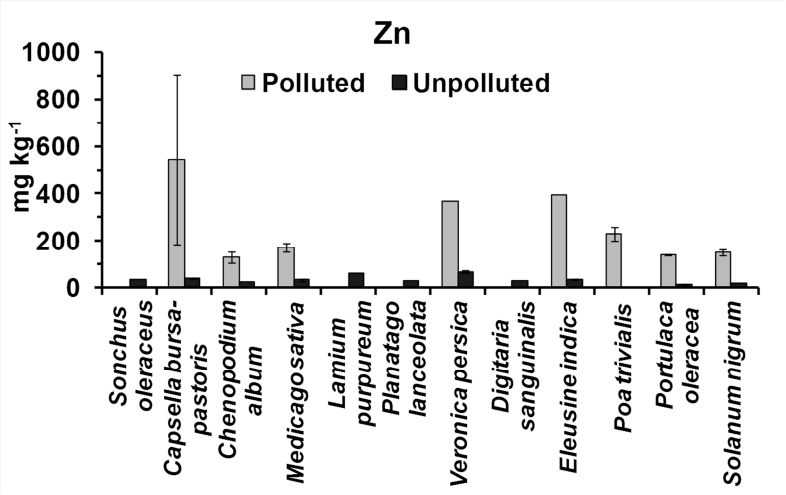
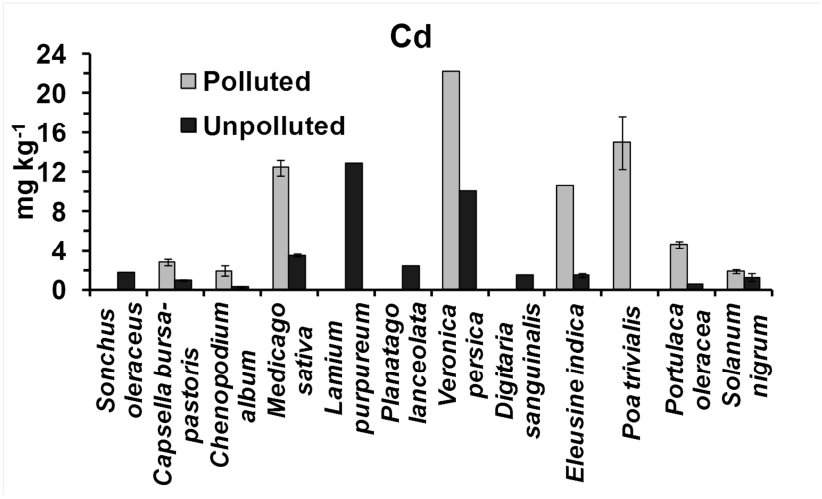


Figure 13. Shoot metal concentrations of spontaneous species growing in the polluted soil. Vertical bars represent standard error.

In the polluted soil and at shoot level, the highest concentration of Cd (8.67 mg kg^{-1} dw), Co (6.38 mg kg^{-1} dw) and Zn (543 mg kg^{-1} dw) were found in *Capsella bursa-pastoris* L., whereas the highest value of Cu (662 mg kg^{-1} dw) was found in *Veronica persica* L.. Relatively high concentrations of metals were also found in *Poa trivialis* L. (Cd, Co, and Zn) and *Eleusine indica* L. Gaertn (Zn). A part from some species, shoot Cd, Co, Cu and Zn concentrations were generally higher when plants grew in the polluted soil than in the reference uncontaminated one.

In the unpolluted soil at shoot level, *Lamium purpureum* L. reached the highest concentration of Cd (2.85 mg kg^{-1} dw), Co (1.41 mg kg^{-1} dw) and Cu (45.41 mg kg^{-1} dw). The highest Zn was found in this species (61.18 mg kg^{-1} dw) along with *Veronica persica* L. (68.90 mg kg^{-1} dw).



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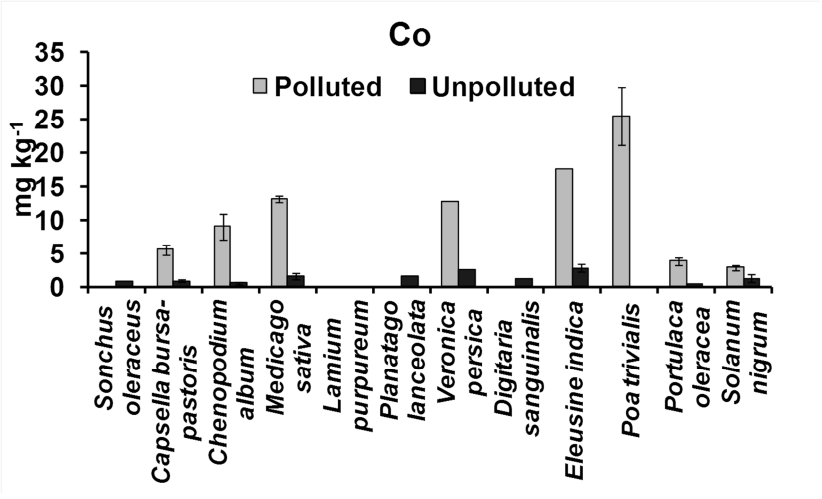

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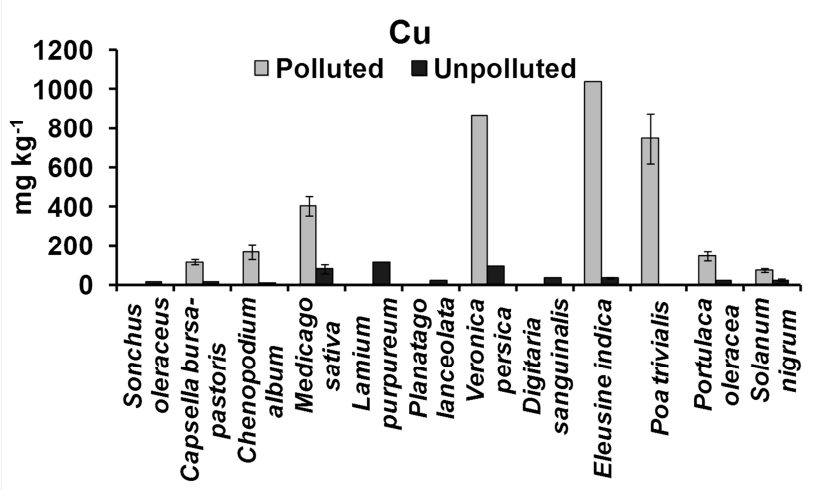

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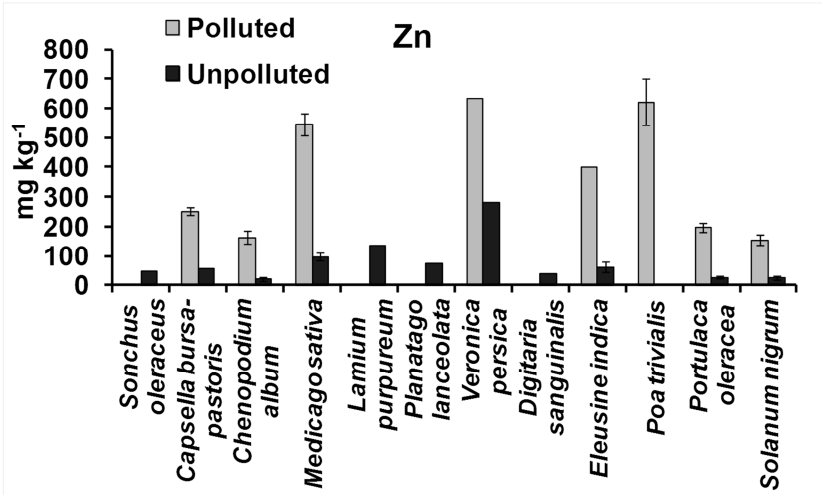
D 

Figure 14. Root metal concentration of spontaneous species growing in the polluted soil. Vertical bars represent standard error.

At root level (Fig. 14), the highest concentration of Cd (22.2 mg kg^{-1} dw) and Zn (635 mg kg^{-1} dw) were found in *Veronica persica*, whereas the highest Cu (1042 mg kg^{-1} dw) and Co (25.50 mg kg^{-1} dw) in *Elusine indica* (L.) Gaertn. and *Poa trivialis* L., respectively.

In the unpolluted soil, *Lamium purpureum* L. reached the highest concentration of Cd (12.87 mg kg^{-1} dw), Cu (117 mg kg^{-1} dw) and Zn (137 mg kg^{-1} dw), while the highest concentration of Co (2.85 mg kg^{-1} dw) was found in *Elusine indica*(L.) Gaertn..

Portulaca oleracea L., which was one of the most abundant species, concentrated small amounts of all metals at both shoot and root level and irrespective of the soil contamination.

2.3.9. Climatic conditions during the trial

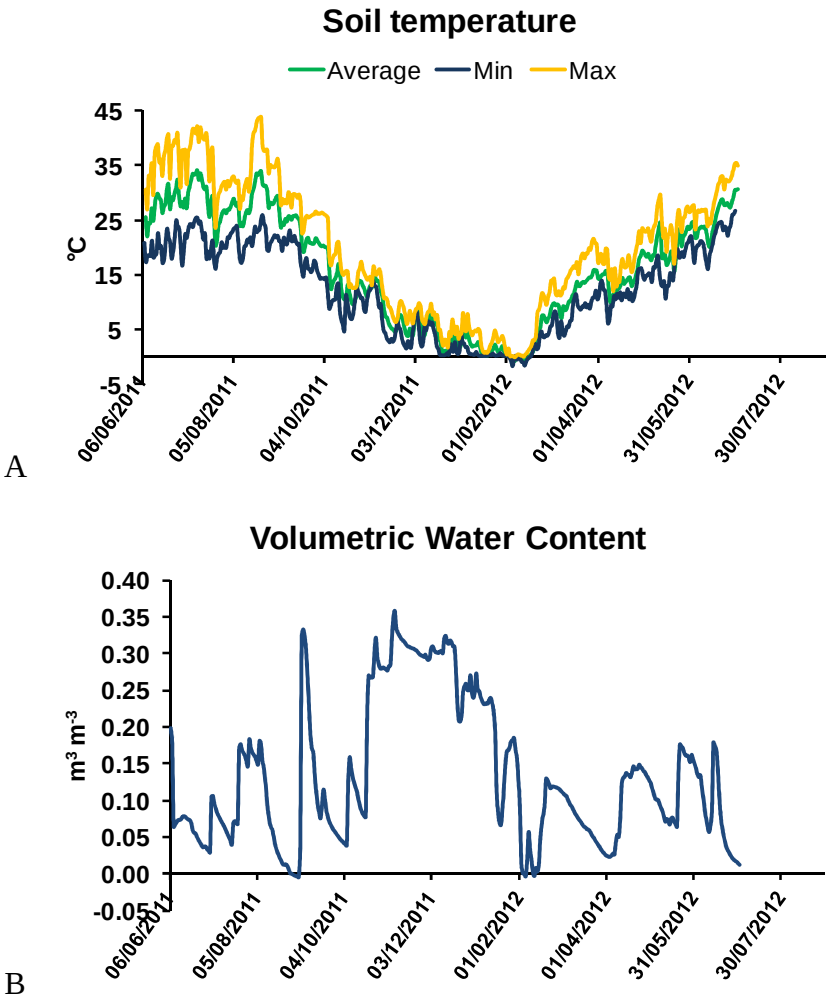


Figure 15. Maximum, minimum and average daily temperature (A) and volumetric water content (B) in the soil during the root degradation experiment.

Soil temperature reached high values at the beginning of the experiment, and the highest temperature (mean 34 °C and Maximum 42.2 °C) was found on the July 12 2011. Then temperature gradually decreased and reached a minimum (mean -0.3 °C and minimum -1.7 °C) on February 14 2012. In summer, the difference among the average, minimum and maximum temperatures was higher than in winter.

Soil moisture was higher in winter, when the temperature were low, due to the low soil water evaporation.

2.4. Discussion

2.4.1. Biomass production and dynamic of taproot degradation

Biomass production is one of the main factors affecting the effectiveness of phytotechnologies, and higher yields generally improve metal removals by biomass species (McGrath *et al*., 2002; Vamerali *et al*., 2012), although also metal concentration in plant tissues plays a relevant role (McGrath *et al*., 2002).

The higher shoot and root biomass production both per plant and per hectare found in the vigorous hybrid Excalibur compared to the semidwarf hybrid PR45D01 confirms that intra-specific variability can be an important source of variation for improving the efficiency of plant-based technologies. Sowing density was also an efficient agronomic tool to improve rapeseed growth, with higher shoot and root productivity (t ha^{-1}) being predicted at elevated densities. These results, together with the higher metal concentrations, at least in roots and seldom in shoots of Excalibur, suggest that specific genotypes properly cultivated can allow a more profitable application of metal phytoextraction and phytostabilization.

In planta phytostabilization requires a long-term immobilization of pollutants within root matter, and this seems more practicable with woody species and polyannual herbaceous plants, whereas for annual ones there probably is some criticism. The decomposition of plants residues in soil is obviously affected by the chemical composition of the litter itself (Swift *et al*., 1979), and different species have varying C content in their matter that affects the amount of energy recoverable by microbes (Swinnen *et al*., 1995). Other variables (Heal *et al*., 1997) are the litter N content (Yavitt and Fahey 1986) and the C:N ratio (Edmonds 1980), together with environmental factors such as temperature and precipitation (Aerts, 1997; Berg *et al*., 2000). The lignin content of degrading tissues has also a strong regulating effect (Gholz *et al*. 1985). In this experiment the latter did not highlighted any substantial differences among treatments, but the root degradation pattern followed by the two genotypes diverged at the beginning of the process. The initial biomass was different between Excalibur and PR45D01, as well as the residual biomass, but it was not so in terms of degradation rate. The initial pattern of biomass loss was different between cultivars, but overall not enough to cause different degradation patterns. The similar root composition among treatments supports the hypothesis that the dynamics of

roots degradation is very stable in terms of fraction of initial biomass. Compared with a previous experiment, root degradation was much slower, suggesting difficulties in predicting the dynamics of litter degradation only referring to its chemical composition (Paustian *et al*., 1997). There was also a strong difference between the two experiments, as the first was carried out in the open, whereas this one was set up in confined boxes that have probably reduced the root degradation rate. Our study suggests that the intraspecific variability and sowing density can only affect the amount of biomass produced by rapeseed.

Other authors reported different degradation rates among various species due to different litter composition (Henriksen and Breland 1999b) and cell types (Chesson *et al*., 1997). Indeed, in this study it was found faster cellulose degradation than lignin, since at the end of the experiment the percentage of cellulose was lower than initial values, whereas those of lignin and ash (AIA) were increased. These results are supported by differential microbial activity measured by the "ferimeters", especially in the second part of the experiment. It is likely that different molecules are degraded either at different rates or at different times. Microbes possibly attack first the most labile compounds (non-structural carbohydrates, aminoacids, peptides etc.), whereas the most recalcitrant ones would be degraded later; this would explain the lower degradation of the cotton threads (cellulose) compared with those of silk (protein) in the first part of the experiment, and the opposite trend in the following period.

All the analysis on degrading roots (e.g., % of residual biomass, FDA, fertimeters) are consistent with the hypothesis that the degradation was faster in the unpolluted soil than in the reference uncontaminated one, due to variation in intensity of microbial activity, despite marked biomass variability of buried roots at the beginning of the experiment.

Within nylon net bags root degradation was probably affected by altered contact between organic matter and soil (Henriksen, 1998), but all the data are affected by the same bias, allowing correct evaluation of the influence of factors under study.

C and N contents are recognized as major predictors for determining degradability of shoot litter (Henriksen and Breland 1999a), but this experiment on root matter innovatively suggests that degradation is also influenced by pollutants via

modulation of microbial activity. An accurate evaluation of the degradation process should therefore take into account soil properties, since they might be an important source of variation in the patterns of litter degradation.

Root degradation followed a sigmoidal model (MMF) that accurately mirrors the loss of weight in both this and the previous experiment within at least 1- or 2-year period. Interpolation of data was performed over time as days after burying, a method only apparently wrong compared with modeling over thermal time. This choice, that did not compromise treatment comparisons, was supported by difficulties in identifying a minimal temperature for blocking microbial activity. Furthermore, this obviously is a simple model that does not consider successive plant cultivation cycles, a condition that would require some adjustments especially if the most recalcitrant root biomass follow a different dynamics.

Efficient management of phytostabilization requires to slow down root degradation after shoot harvest (e.g. phytoextraction) or plant death. In this regards, the agricultural means that were tested, genotype selection and sowing density, had not any significant influence, although they allowed to increase rapeseed productivity. Therefore, it was concluded that to maximize over time the stock of organic root material the initial productivity must be as high as possible.

2.4.2. Effects of metals on spontaneous species

Soil contamination by heavy metals affected root degradation, as well as the composition of the spontaneous flora. Most of the species were found in both the polluted and unpolluted soil, probably because many of them might tolerate high levels of pollution. This confirms literature results and suggests that native plants might be useful for both *in-planta* and *ex-planta* metal stabilization through uptake and reduction of soil erosion (Mendez and Maier, 2008). The high concentrations of HM found in *V. persica*, *P. lanceolata*, *E. indica*, and *P. trivialis*, also suggests that spontaneous plants belonging to *Plantaginaceae* and *Poaceae* families might be investigated for the accumulation of HM in both shoot and root. Particularly high was Cu concentration in both shoots and roots of *V. persica* L., a species previously ascribed as belonging to *Scrophulariaceae* family, and only recently classified into

Plantaginaceae. On the contrary, the low concentration of heavy metals in *Portulaca oleracea* L., irrespective of the soil pollution level, suggests that this species might be a metal-excluder.

Metal concentrations were generally much higher in roots than in the shoot, a common result in many biomass species caused by reduced translocation and that suggest to exploit both phytoextraction and phytostabilization processes.

2.4.3. Heavy metal uptake and dynamic of metal releasing

The higher concentrations of Cd and Cu in roots of Excalibur than in those of PR45D01 and the higher accumulation of all the metals at elevated plant density suggest that genotype selection and sowing density can significantly affect the overall accumulation of HM. Intraspecific variability deserves to be exploited and agronomic practices to be tuned for achieving high remediation potentials. In this way, the hybrid Excalibur at 63 plant m^{-2} resulted the best choice to accumulate a high stock of pollutants in the shoot for phytoextraction purposes and in the taproot for phytostabilization.

Since metal concentrations and residual root biomass were always the same irrespective of the genotype, Excalibur was not able to maintain a higher metal stock, and the same happened for the main effect density. Surprisingly the loss of root biomass was accompanied by increasing metal retention, a fact due to increased metal concentrations in root matter. In this model-species (rapeseed) metals are probably stored in both easily degradable and recalcitrant tissues, as in the previous experiment there was a clear reduction in the root metal stock. A relatively high concentration of metals in degrading tissues is of course expected as one important metal sink in plants is represented by the cell wall (Manara, 2012), which is largely composed by recalcitrant compounds like cellulose and lignin. In this regards, according to the results of a previous experiment, after one year of incubation the residual content of metals was expected to be relatively high, depending on the element. Instead, unexpected was the sharp increase over time in total metal contents (mg per hectare) in degrading roots, which is also in contrast with the previous results in uncontaminated soil. Increases in metal concentration in the dead biomass

agrees with evidence that plant-derived biomass is able to adsorb heavy metals from industrial effluent (Verma and Shukla 2000; Prasad and Freitas, 2000). Roots may adsorb metals onto their surfaces through ion exchange and other mechanisms (Schneider *et al*., 2001), especially in the polluted soil where the bioavailability of HM was found very high. It should also be pointed out that the previous experiment was conducted in the open, where soluble metals can be leached downward, whereas this experiment was conducted in closed boxes (to avoid soil contamination), where no leaching could occur and the soluble metals were therefore available for adsorption.

The high metal bioavailability in the polluted soil might explain higher metal contents in the degrading roots. Moreover, after intense precipitation the amount of soluble metals is expected to greatly increase due to water stagnation in boxes, thus further increasing the amount of soluble metals available for adsorption onto root matter. These results suggest that, although the accumulation of metals during the growing season is affected by the genotype and density (other than by the chemical properties of the soil that affect metal speciation and bioavailability), the residual contents of heavy metals in degrading roots might be more affected by the soil conditions; therefore, it was hypothesized that the initial metal contents might be less important in determining the rate of adsorption/release.

The fact that each metal followed its own trend is consistent with the results of the previous experiment and confirms that different metals might be released/adsorbed at different extent depending on their bioavailability, with higher adsorption at elevated metal mobility. The adsorption of heavy metals onto plant-derived biomass is usually modeled through the Langmuir isotherm (Wang, 1995; Wang *et al*., 1998, Schneider *et al*., 1999), therefore the trend proposed here don't agree with the available literature about adsorption of heavy metals from aqueous solutions onto dead biomass. The estimated model of metal release/retention in this root matter was often sigmoidal, and efficiently represented the phenomena for all the 'metal × soil' combinations, suggesting that other models (other than the Langmuir model) might be used to describe the interaction between metals and dead biomass. More difficult was the Cu modeling in the unpolluted soil, a fact probably due to the natural high affinity of this metal for organic matter.

Copper and Cd were the only metals with a negative tendency of their stock in root matter in the unpolluted soil, confirming that metals are released during the degradation despite adsorption onto root surfaces, probably because of different equilibrium conditions in the soil.

2.5. Conclusion

Litter degradation in soil has been studied for decades, but at present it is still difficult to explain the process due to the high number of variables (environmental and genetic) potentially involved; on the contrary, the potential effects of litter degradation on efficiency of *in-planta* phytostabilization of heavy metals has not been investigated at all. It is known that in many spontaneous and cultivated species fine root turnover is a fast process which involves a large part of roots already within the growing cycle, but there is not information on dynamics of taproots degradation of annual species after above-ground harvest or plant dead.

This study, although far from explaining the whole process, highlighted that the degradation of root litter in soil is clearly affected by soil contaminants, due to a reduction of the microbial activity responsible for the degradation of plant residues. The initial biomass produced and the amount of HM accumulated in roots set the metal stock potentially releasable as a consequence of degradation. We think therefore that the initial metal contents should be maximized for an effective stabilization through species and genotype selection and appropriate sowing density, but this amount can be further increased over time through the absorption capacity of the litter especially under high metal bioavailability. Soil characteristics may result more important than those of the residue for stabilization of heavy metals.

Here the residual heavy metal contents were clearly biased by the presence of the boxes, but the results suggest the possibility to greatly rise the amount of metals retained in organic materials by preventing metal leaching, for instance through impermeable barriers that prevent water and metal movement downward.

To offset biomass losses and reduce metal release, new litter should be periodically added to the soil to enhance metal adsorption, which is expected to improve the process through repeated growing cycles. These results are not definitive

on the fate of root matter, but the still high values of undegraded biomass (about 30% and 40% in unpolluted and polluted soil, respectively) suggest that increasing stock of organic matter and metals are probably achieved through the annual deposition of recalcitrant taproot biomass.

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Chapter 3

Different waste organic amendments increase soil metal bioavailability through variation in dissolved organic matter and humic substances: effects on forage sorghum

Abstract

Worldwide, many soils have been losing their fertility because of remarkable reduction in carbon content. Organic amendments from by-products or wastes from agricultural and industrial activities can be successfully disposed of to hinder the loss of productivity.

In a mesocosm study, the effects of three different organic amendments (mature compost from green and municipal solid wastes; solid fraction of anaerobic digestate from agro-industrial wastes; solid fraction from pig slurry) were assessed on productivity and growth of forage sorghum and on metal accumulation in both soil and plants. The tested amendments were mixed with a silty-loam texture and poor organic matter content soil at a rate of 10 t ha^{-1} of organic carbon, allowing to increase the total content of organic carbon from 0.83 to 1.2%. Compared to the unamended reference soil, above-ground biomass of sorghum (3 cuts) was increased by 26, 11 and 5.8% by compost (CP), pig slurry (PS) and digestate (AD) respectively, in view of their higher nutrient contents, especially nitrogen. Root length density was also increased in CP and AD, due to the hormone-like effects of their humic substances. None of the amendments significantly increased metal concentrations in shoot tissues, although they all increased Zn and Ni bioavailability and AD resulted in a higher total soil Co (+4.9%), Cr (+7%) and Cu (+6.8%) at the end of the cultivation cycle. Increased metal bioavailability (Cd+Cu+Ni+Pb+Zn) was associated to the high dissolved organic matter (DOM) of pig slurry and

digestate through formation of soluble metal complexes, whereas the high humic substance of compost prevented metals from becoming more soluble. It is concluded that matured organic waste exerts more favorable agronomic effects without rising environmental or health risks, at least in the medium-term. Compost seems also offer more recalcitrant organic matter to degradation, although possible soil contamination by heavy metals should be evaluated for each waste batch.

3.1. Introduction

Agriculture plays the paramount role of producing foods, but many soils worldwide are losing their productivity because of overexploitation. Soil quality is related to its chemical, physical and biological characteristics, and is referred as soil fertility, i.e., the ability to sustain crop growth by supplying nutrients and water (Giardini, 2008). Soil organic matter (SOM) is a key factor in plant growth and productivity as responsible of several favourable soil properties. It is a stock of nutrients, enhances air and water movements and reduces soil compaction (Hamblin and Davies, 1977), stimulates microbe activity (Clark *et al*., 2007; Jones and Healey 2010; Carter, 2002). The most important fraction of SOM is represented by humic substance (HS), a class of organic compounds within 10000-100000 Da molecular weight, insoluble in acidic conditions which is responsible for the benefits of SOM itself (Stevenson, 1994).

Common agricultural practices like tillage, intensive monoculture and the use of mineral fertilizers instead of manure have caused severe soil organic matter reduction, highlighting the need of preserve and increase the carbon stock under a new view of sustainable agriculture.

In recent years, organic amendments from urban and agro-industrial wastes have been used as replacement of manure or inorganic fertilizers. This approach has the advantage of improving soil quality, reducing landfill disposal of wastes and meeting European environmental policy that aims to increase the recycling of biodegradable wastes (Smith, 2009). However, there also are some drawbacks such as soil salinization (Rodgers and Anderson, 1995) and pollution by heavy metals and other contaminants. In fact, total and bioavailable Cd, Cu, Pb and Zn are reported to

increase (Illera *et al*., 2000; Ramos and López-Acevedo, 2004; Farrell *et al*., 2010), rising doubts about the environmental compatibility of such amendments because of possible leaching and accumulation in the food-chain. However, in other studies it was found that organic materials can induce metal(loid) retention due to the presence binding compounds (Gondar and Bernal, 2009). Sewage and paper mill sludge (Merrington *et al*., 2003; Sajwan *et al*., 2003), green and municipal solid waste compost (Alvarenga *et al*., 2009) and cow manure (Narwal and Singh, 1998) have been found to reduce soil metal bioavailability. It has recently suggested that organic amendments like chicken manure (Wei *et al*., 2010) and many other organic wastes (Clemente *et al*., 2006; Jones and Healey, 2010) can be used to stabilize heavy metals in contaminated soils in phytoremediation.

In this framework, this study aimed at assessing the effects of supplying a fixed amount of organic carbon (10 t ha^{-1}) from various waste organic amendments differing for the feedstock material and maturity (humification rate) to a organic matter poor soil. It was verified if the different source of organic matter has an influence on i) shoot and root growth of forage sorghum, ii) soil total and bioavailability metals and iii) metal accumulation in plants. Improved knowledge was also achieved on possible mechanisms of metal mobilization/retention through analysis of dissolved organic matter and humic substances.

3.2. Material and Methods

3.2.1. Experimental set up

The experiment was carried out in large pots (mesocosms) at the Stuard experimental farm of Parma during 2010 growing season. Soil analysis at the beginning of the experimental trials, cultivation and biomass measurements were performed at the University of Parma, while soil coring for root analyses and heavy metal contents in both soil and plant biomass were performed at the University of Padova.

The experimental soil had very low Total Organic Carbon (TOC) content (0.83%), and was not fertilized for one year before experiment. The soil, collected at

Alfonsine (Ravenna, Italy), was classified as *Calcaric-Cambisols* with silty-loam texture. Its chemical characteristics before and after amendment are listed in Table 1.

Table 1. Main soil characteristics at the beginning of the experiment in reference unamended control (T) and in amended treatments (CP = Soil + Compost; PS = Soil + Pig Slurry; AD = Soil + Anaerobic Digestate).

Parameter		Soil (T)	CP	PS	AD
pH	□	8			
Texture (Sand, Silt, Clay)	%	4, 46, 20	-	-	-
Total Lime	%	22	-	-	-
CEC	$Cmol^{+}/100g$	14.5	-	-	-
TOC	% dw	0.83	1.07 (+28%)	1.12 (+35%)	1.07 (+28%)
Total N[(1)]	g kg^{-1} dw	1.07	1.32 (+23%)	1.27 (+19%)	1.18 (+10%)
Total P	g kg^{-1} dw	0.62	0.70 (+13%)	0.76 (+22%)	0.65 (+4.0%)
Humic and Fulvic Acids (HA + FA)	% dw	0.27	0.37 (+36%)	0.30 (+11%)	0.32 (+17%)

[1]Kjeldhal

The experimental soil was collected on December 12 2009 from the 0-30 cm depth horizon and air-dried in greenhouse. On January 19th 2010, the soil (~ 1 t) was divided into four aliquots, three of which were mixed with either i) mature compost from municipal solid wastes, green wastes, and agro-industrial wastes (CP), or ii) solid fraction from pig slurry (PS), or iii) solid fraction of anaerobic digestate from green and agro-industrial wastes (AD). The amount of amendments were calculated on the basis of their humidity and composition to add a fixed amount of organic carbon (10 t ha^{-1}), in comparison with a unamended reference control (T). Chemical and physical characteristics of the amendments are listed in Table 2.

A mesocosm experiment was set-up after mixing soil with either compost, or digestate or pig slurry by filling large pots (height 0.35 m, 0.38 m diam.) with ~60 kg of amended soils (only soil for controls), following a completely randomized

experimental design (n = 3). Before pot filling, a 4-cm clay layer was placed at the bottom of each pot to avoid water stagnation. The pots were placed within a greenhouse which had an automatic opening system to keep temperature very similar to ambient temperature.

Table 2. Main chemical characteristics of organic waste amendments.

Parameter		Comopst	Pig Slurry	Anaerobic Digestate
pH	-	8.56	6.75	8.66
Conductivity	mS cm^{-1}	2.01	1.6	2.49
Water content	%	31.37	53.63	72.42
Dry Matter (DM)	% fw(1)	68.63	46.37	27.58
Loss of Ignition (LOI)	% fw	22.89	38.92	24.5
Total N(3)	ng kg^{-1} dw(2)	19746	31419	26933
N-NH_4	mg kg^{-1} dw	1424	623	6407
Total P	mg kg^{-1} dw	6392	21533	6294
Total K	mg kg^{-1} dw	12581	9352	20812
Cu	mg kg^{-1} dw	109	135	12
Zn	mg kg^{-1} dw	262	281	140
TOC	% dw	18.86	45.89	58.53
HA+FA	% dw	7.89	4.96	11.47

(1) fresh weight; (2) dry weight; (3) Kjeldhal

Cv. Grazer-N of sorghum (*S. bicolor* (L.) Moench × *S. sudanense* (Piper) Stapf.*)* was sown in each pot at a density of 258 seeds per m^{-2} (36 seeds per pot) on April 13 2010 and regularly irrigated to maintain water content at 20-25% w/w (field capacity at 35% w/w). Plants were grown over 5 months providing three biomass harvests (cuts) (June 10, July 20 and October 6 2010). After the last cut, three soil core were collected from each pot and used for soil and root analysis.

3.2.2. Plant growth and metal analysis

Aboveground biomass

Sorghum plants were harvested when reaching ~80-100 cm height and water content was < 70%. Shoot fresh and dry (oven-drying at 105 °C for 24 hours) weights were measured before elementary analysis (Cd, Co, Cr, Cu, Mn, Ni, Pb and Zn). Metals were revealed through ICP-OES (Inductively Coupled Plasma-Optical Emission Spectroscopy) after microwave acid digestion (Microwave Labstation, Ethos 900) following the USEPA (1995b) method.

Root Growth

At the end of the experiment, root length density (RLD) was measured through destructive sampling by collecting two 53-mm diameter 300-mm long cores from each pot. They were subdivided into three 10-cm long subsamples. Roots were separated from soil through flotation by means of a hydraulic centrifugation device and collected in a 500-µm mesh size sieve, as described by Oliveira *et al*., 2000. Separation of soil particles was facilitated by the addition of a 2% w/v oxalic acid solution. Roots were stored at 4 °C in 12% v/v ethanol solution until analysis. One-bit 400-DPI TIFF format images of roots were acquired by digital scanning on a flet-bed scanner (EPSON Expression 10000XL, Canada). Root length was measured by the KS 300 image analysis software (Carl Zeiss Vision GmbH, München) following the procedure of Vamerali *et al*., (2003a). Discrimination of roots against extraneous objects was performed according to an elongation index value (i.e. perimeter^2 /area) > 60 and minimum object area >25 pixels. Root length (FbL) was derived from perimeter (P) and Area (A) of digital objects, as follow:

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

Root length of each subsample was referred to its soil volume for determining the volumetric root length density (RLD, cm cm^{-3}).

3.2.3. Soil Analyses

Carbon content, Dissolved Organic Matter and Humic Substances

Dissolved organic matter (DOM) and Humic Substances (HS) carbon contents (Walkley and Black 1934) were measured at 0-15 cm depth after the last biomass cut.

DOM was extracted from air dried soil samples using double deionized water with an extraction ratio of 1:2 w/v (15 g in 30 mL) (Corre *et al*., 1999). The suspension was shaken for two hours at room temperature in enriched N_2 atmosphere and then centrifuged at 7000 *g* for 5 min. Extracts were filtered on microfiber glass filters (Whatman, Maidstone, England), then on nylon 0.45 µm filters (Millipore, Milford, MA, USA).

HS were extracted from air dried samples using a 0.1 M KOH solution with a solid:volume ratio of 1:10 (10 g in 100 mL) as described by Carletti *et al*. (2009). DOM and HS extracts were stored at -20 °C until analysis. Organic carbon content was essayed by dichromate oxidation (Walkley and Black, 1934). Molecular-weight distribution and gel-permeation chromatography of each humic extract was carried out on a Sephadex G-100 gel packed in a 70×1.6 cm Pharmacia column (Pharmacia, Uppsala, Sweden). The gel packing and the mobile phase were represented by a 20 mM $Na_2B_4O_7$ Solution. The apparent molecular weight of the fractions were separated into three classes, i.e., >100, 100–10 and <10 kDa. The column calibration (Kit MS-II, Serva, Heidelberg, Germany) was based on previously assessed standard proteins (Carletti *et al*., 2009).

Part of the humic extracts were transferred into Visking tubing (14 000 mol. wt cut-off; Medicell, London, UK) and dialyzed against double-distilled water. The water was changed daily until the liquid outside the dialysis tube was colorless. The retained solutions was desalted by ion exchange on Amberlite IR 120 H^+ and stored for the subsequent bioassays.

The residual TOC at the end of the cultivation cycle was also measured on air dried soil (Walkley and Black, 1934) at 0-15 cm depth.

Hormone-like activities bioassay for humic substances

The auxin and gibberellin-like activities of the DOM were assessed by checking the growth reduction of water-cress (*Lepidium sativum* L.) roots and the increase in the length of lettuce (*Lactuca sativa* L.) shoots, respectively (Audus, 1972).

Water-cress and lettuce seeds were surface-sterilized by immersion in a 8% v/v hydrogen peroxide for 15 min. After rinsing 5 times with sterile distilled water, 10 seeds were aseptically placed on filter paper in a sterile Petri dish (Pizzeghello *et al*., 2006). For water-cress, the filter paper was wetted with 1.2 mL of 1 mM $CaSO_4$ (control); or 1.2 mL of 20, 10, 1 and 0.1 mg l^{-1} indoleacetic acid (Sigma) to obtain the calibration curve; or 1.2 mL of a serial dilution of the HS extract into 1 mM $CaSO_4$ solution. For lettuce, the experimental design was the same as for water-cress, except that the sterile filter paper was wetted with 1.4 mL of the above solutions and the calibration curve was a progression of 100, 10, 1 and 0.1 mg l^{-1} gibberellinic acid (GA) (Sigma).

The seeds were germinated in the dark at 25 °C. After 48 h for watercress and 72 h for lettuce, the seedlings were removed and the root or shoot lengths were measured with a digital gauge. Root (water-cress) and shoots (lettuce) growth data were standardized against the respective data of the controls. The values obtained were the means of 20 samples and five replications (Ertani *et al*., 2011), and standard errors were always <5% of the mean

Heavy metals

At the end of the experiment, pseudo-total arsenic and metal concentrations in soil samples were measured after mineralization following the USEPA (1995a) method.

DTPA-extractable metals (Cd, Cu, Ni, Pb and Zn) were also measured to asses metals bioavailability (Lindsay-Norwell 1978).

3.2.4. Statistical Analysis

All data of biomass weight and total and bioavailable heavy metal concentrations were analyzed by ANOVA after checking for normality and homogeneity of variances. The Bonferroni or Student-Newman-Keuls ($P \leq 0.05$) test was performed for biomass and metals, respectively, in order to highlight differences among means. When ANOVA assumptions were not satisfied, the non parametric Friedman test was performed and significant differences were highlighted through pairwise comparisons through the Wilcoxon test ($P \leq 0.05$).

All statistical analyses were carried out with Costat Software (Cohort, Monterey, CA, USA) for ANOVA, and XLstat (Addinsoft, Paris, F) for the non parametric test.

3.3. Results

3.3.1. Carbon and nutrients after amendment

The addition of organic amendments was planned to rise the total organic carbon (TOC) content from 0.83% to about 1.08%, and this goal was achieved at the beginning of the experiment for CP and AD, whereas a slight greater TOC (1.12%) was detected for PS (Table 1). Anyway, the higher TOC of PS compared with the other treatments, that was attributed to some water loss of pig slurry during transport from the pigpen, was considered negligible.

At the end of the experiment, TOC had decreased in all treatments (i.e., 1.02, 0.96 and 0.99% in CP, AD and PS respectively) but it was still much higher in the amended soils than in controls (0.73%). The loss of TOC was minimal in CP (-3.9%), the highest in PS (-12%) and intermediate rate in AD(-10.5%). and T (-8.6%)

Humic substances content evidenced a significant increase in the amended thesis compared to untreated controls, with the highest values in the CP (+36%) and AD (+17%) treatments (Table 1). As regards nutrients, with pig slurry it was reached the highest increases of P (+22%) together with and a substantial N improvement (+19%) (Table 1). With compost we detected the maximum increase

in total soil N (+23%) encompassed with a good enhancement of P (+12%), whereas the anaerobic digestate had had lower both N and P, especially for the latter which did not differ greatly from controls (Table 1).

3.3.2. Plant growth

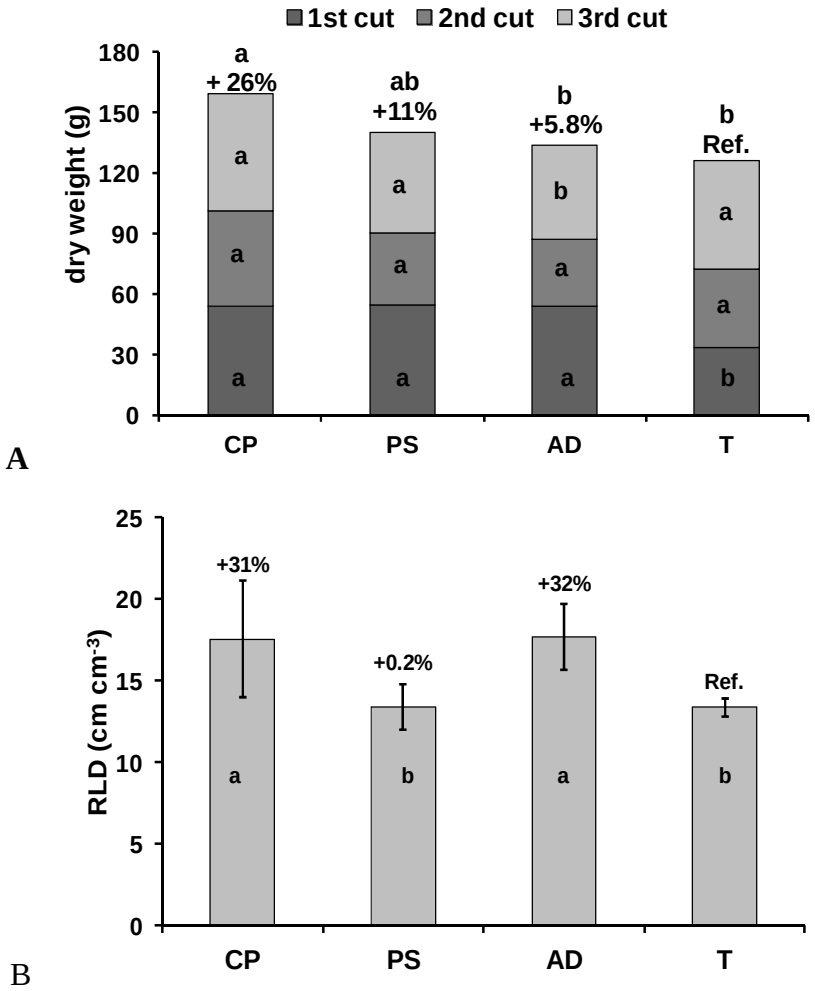


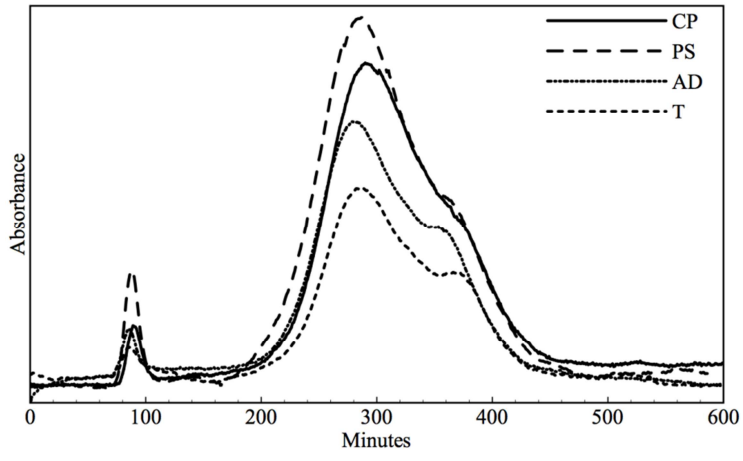
Figure 1. Shoot dry biomass (g per pot) at each harvest and percentage increase in the total biomass produced throughout the season (sum of the three cuts) (A) and Root Length Density (RLD) in the 0-30 cm depth soil layer (B) compared with unamended controls. Vertical bars represent standard error. Different letters indicate statistically significant differences among treatments (for shoots capital letters refer to overall biomass) (Student Newman-Keuls test, $P \leq 0.05$).

Both above-ground biomass and final root length density (RLD) were positively affected by the addition of organic amendments. Differences in shoot growth were mainly observable at the first and last forage cuts, with great advantages

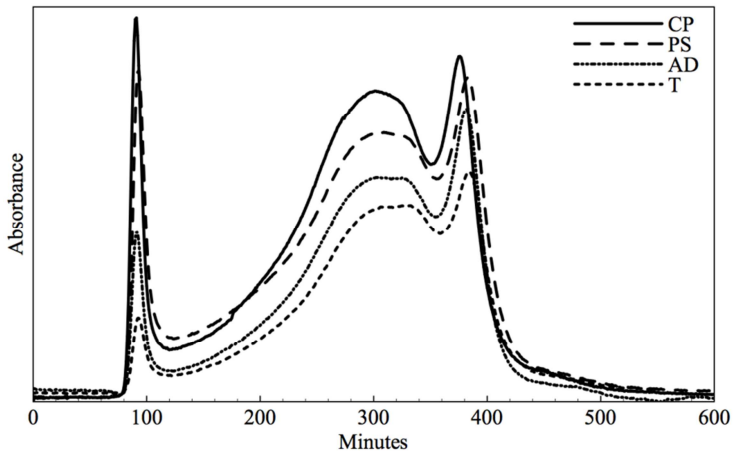
in productivity with organic amendment especially at the beginning. The total shoot biomass produced over the season (sum of 3 cuts) in controls was 126 g d.w. per pot, corresponding to 8.3 t ha^{-1}, and it was improved by 26% and 11% by compost and pig slurry respectively (Fig. 1A). The initial advantage deriving by amendment with digestate was compensated by lower production at the end of the season, so it did not significantly differed from controls.

Root length density (RLD) did not differ between 0-15 cm and 15-30 cm soil layers (main effect), therefore only the average RLD of the whole profile was considered (Fig. 1B). The mean RLD of unamended controls was 13.37 cm cm^{-3}, and it was significantly increased in CP and AD (+31 and 32% respectively). In contrast, root diameter was very stable among treatments, reaching a mean of 283 µm in T and only slight higher values in the amended thesis (data not shown).

3.3.3. HS and DOM gel permeation chromatography



A



B

Figure 2. Gel-permeation of DOM (A) and Humic Substance (B) extracts.

The molecular weight (MW) distribution in DOM chromatograms shows two well defined peaks corresponding to 100 KDa and 100-10 KDa respectively, and a not well defined peak with <10 KDa apparent molecular weight (Fig. 2A). The water extracts showed the highest amount in the intermediate MW compounds and the smallest in the high MW HS (first peak). The first peak is usually composed by more recalcitrant and hydrophobic molecules, which are less present in aqueous extracts. Compared with controls T, amendment with pig slurry (PS) increased the high and intermediate MW compounds, while AD increased low MW substances with a concomitant decrease in the other peaks (percentage). Compost amendment (CP) resulted in a strong decrease in the first peak, coupled with a slight increase of the second peak (Table 3).

The DOM content was increased in all the amended thesis compared to the control, especially in PS (Table 3).

Table 3. Relative area of the peaks in the DOM gel-permeation and average DOM content in soils at the end of experiment.

	Area (%)			DOM % soil dw
	100 KDa	100-10 KDa	<10 KDa	
CP	2.31	76.14	21.56	0.01021
AD	2.77	73.21	24.02	0.01023
PS	3.85	76.2	19.95	0.01137
T	2.83	74.11	23.07	0.00752

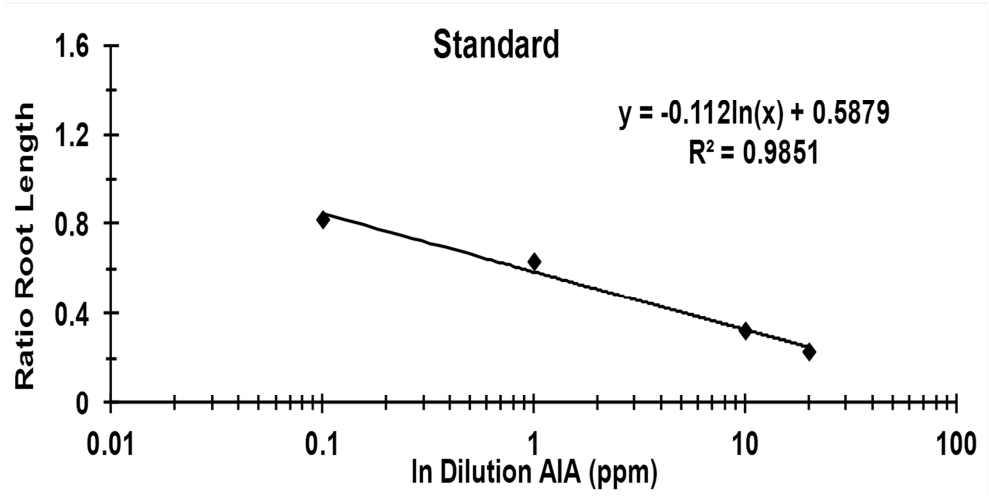
Gel permeation of humic KOH extracts (Fig. 2B) evidenced small effects of the three amendments on the 100-10 KDa MW substances. The highest increase in the first peak was found in the PS as well as in the aqueous extract. Instead, CP evidenced an increase in high MW compounds. In AD amended soil the amount of low MW HS was not significantly different from the control thesis (T).

Table 4. Relative area of the peaks in the HS gel-permeation and average HS content in the soil at the end of experiment.

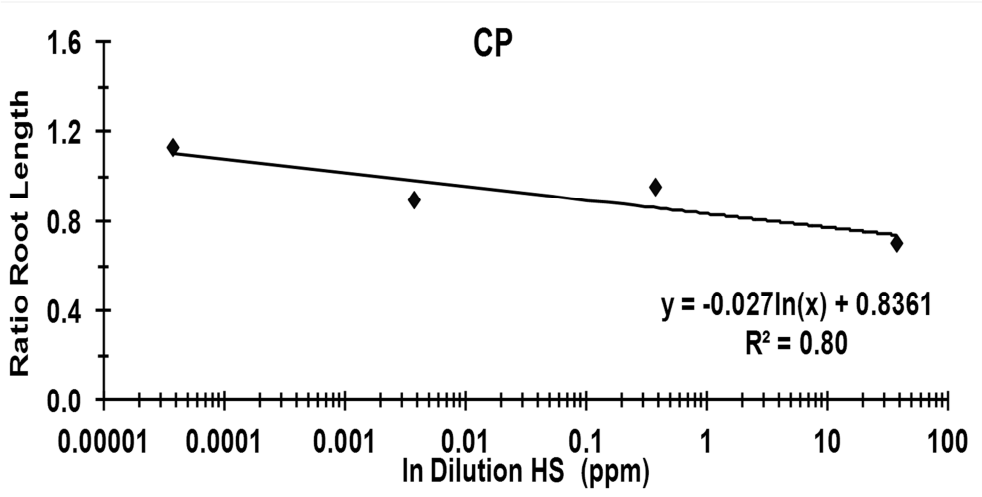
	Area (%)			HS % soil dw
	100 KDa	100-10 KDa	<10 KDa	
CP	8.98	66.98	24.04	3.63
AD	5.66	66.61	27.73	3.67
PS	9.8	65.91	24.3	3.57
T	3.91	68.3	27.79	3.45

Compared with controls, HS content was higher in all the amended soils, especially in AD and CP (Table 4).

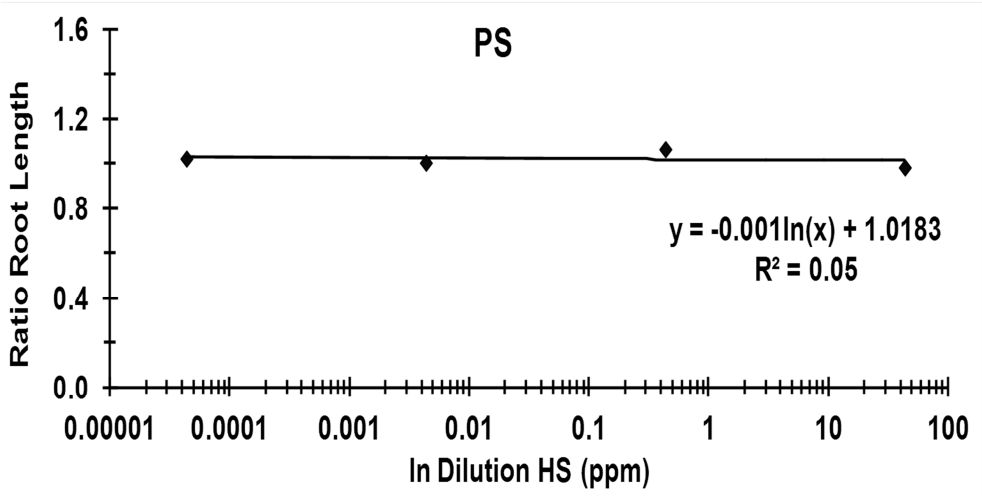
3.3.4. HS hormone-like activity bioassays



A



B



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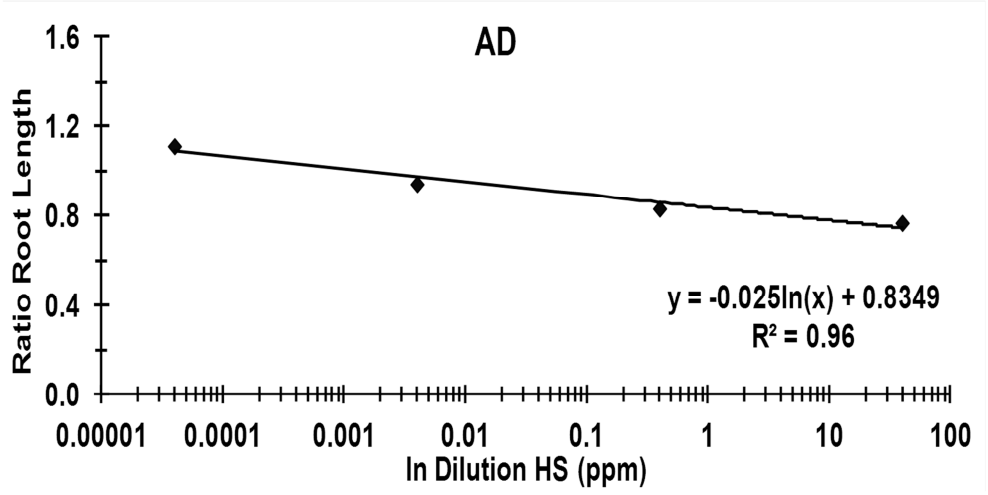

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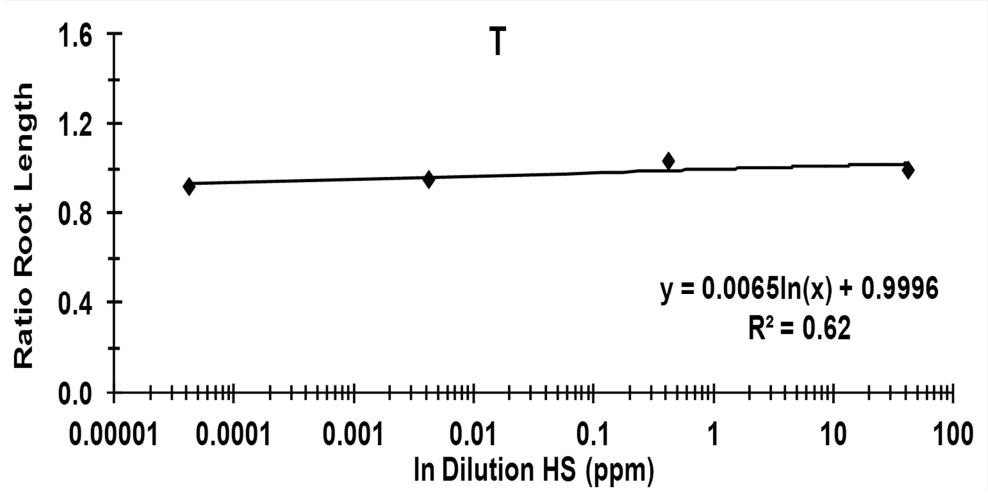
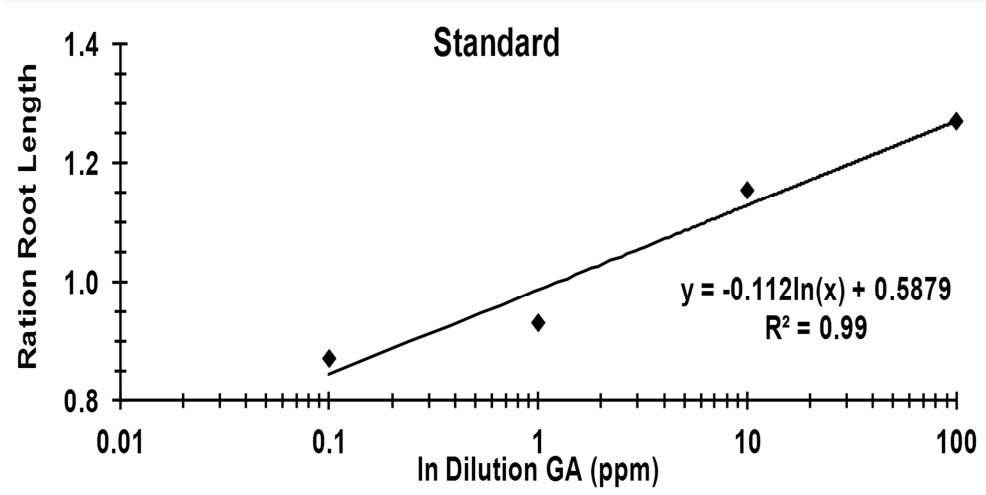


E

Figure 3. Bioassay for Indol-Acetic Acid (AIA) activity in water-cress for different amended soils. AIA or humic substances extract (HS) concentration (logarithmic) in solution is related to standardized (on controls) root length.

A linear regression model was performed to estimate the dose/response between HS extracts and water-cress root growth or lettuce shoot length (Figs. 3, 4). Root growth was negatively correlated with the HS extract concentration of both compost CP (R^2 = 0.80, Fig. 3B) and anaerobic digestate AD (R^2 = 0.96, Fig. 3D), revealing an auxin-like dose-dependent response as evidenced in the calibration curve (Fig. 3A). In contrast, no statistically significant dose-dependent response was induced with pig slurry (PS) and unamended reference (T) soil extracts (Fig. 3C and 3E, respectively).



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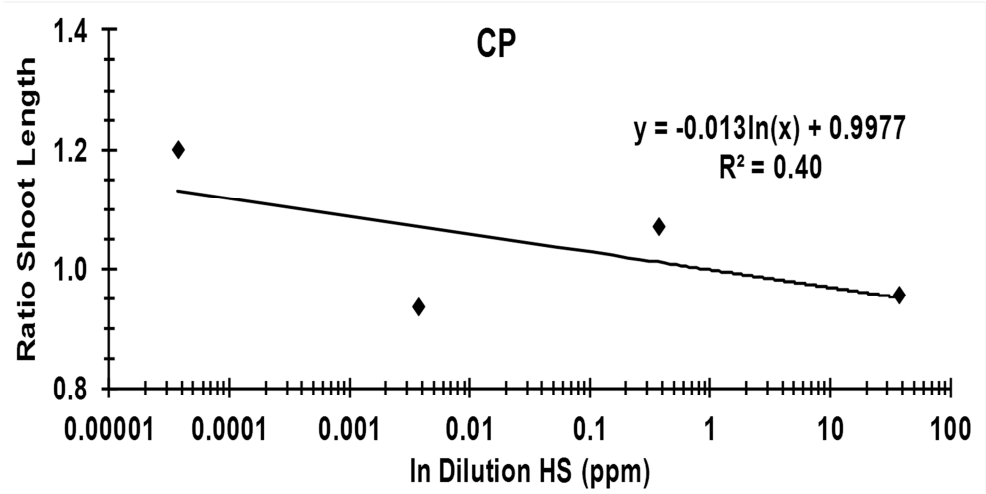

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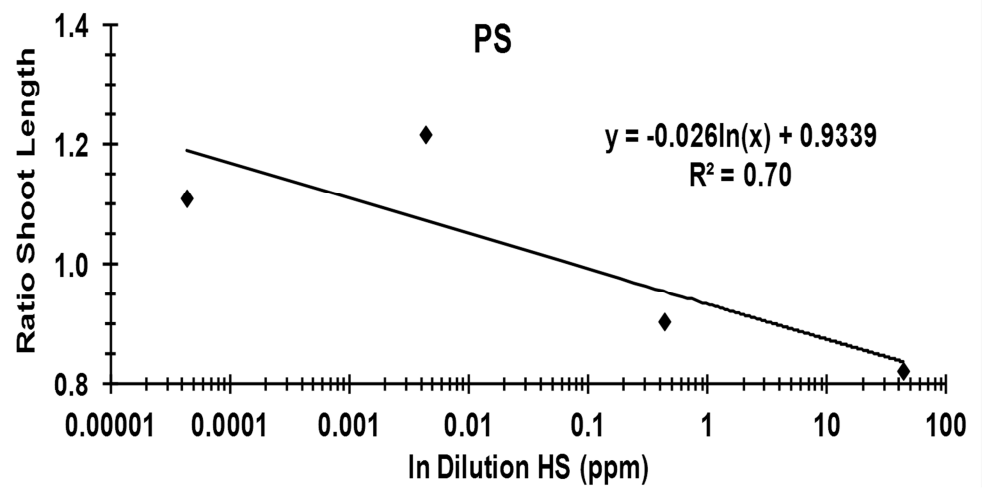

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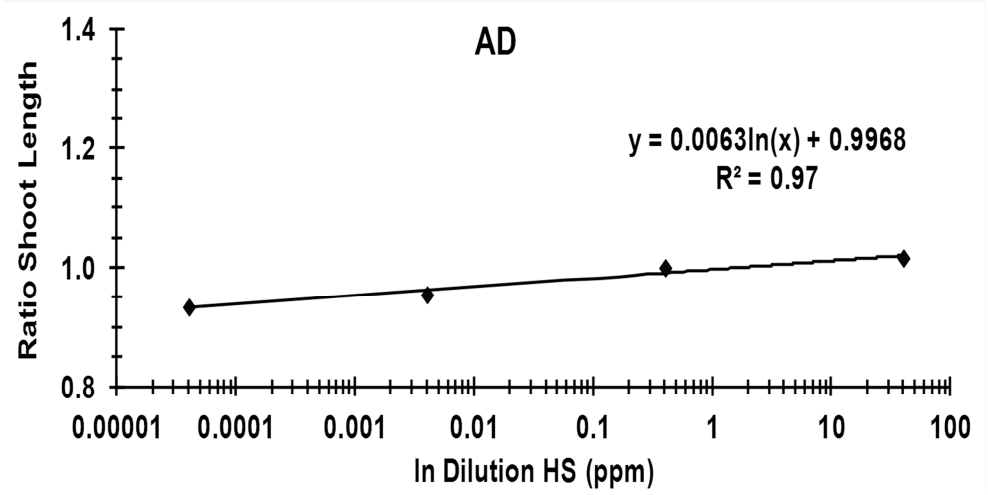

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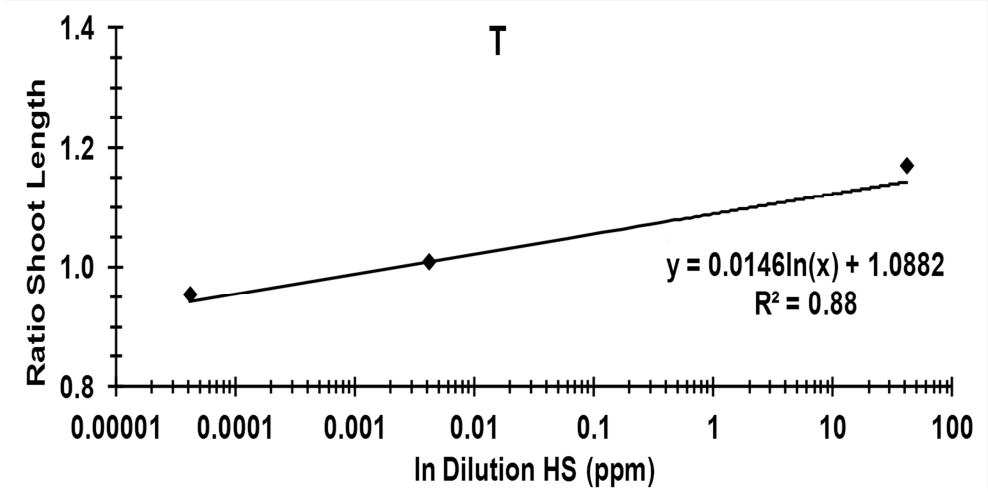


E

Figure 4. Bioassay for Gibberellin Activity (GA) in lettuce for different amended soils. GA or humic substances extract (HS) concentration in solution is related to standardized (on controls) shoot length.

Lettuce shoot length was positively correlated with different concentrations of HS extracts in AD (R^2 = 0.97) and T (R^2 = 0.88) (Fig. 4D and 4E), confirming a GA-like dose-dependent response as in the calibration curve (Fig. 4A). HS extracts from CP and PS treated soils showed absence of GA-like activity (Fig. 4B and 4C, respectively).

3.3.5. Total and bioavailable concentration of heavy metals

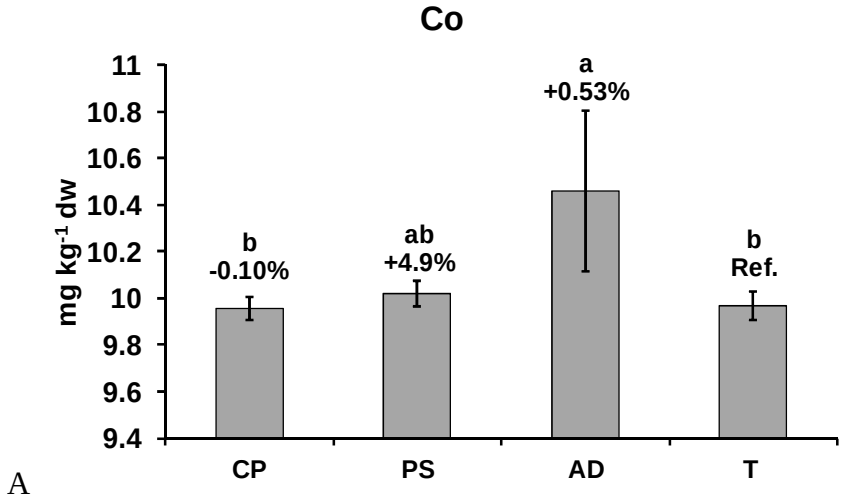
Total and bioavailable metal concentrations did not differed between soil horizons, therefore results are summarized as average of the whole soil profile.

Total metal concentrations did not exceeded the Italian Guideline Values (IGV) for agricultural soil (Ministerial Decree 152/2006) before (Table 5) and after amendment.

Table 5. Italian Guideline Value (IGV) for concentration of heavy metals in agricultural soils set by the Ministerial Decree 152/2006 and total concentration (mean ± S.E., n = 3) in the control soil (T) at the end of the experiment.

mg kg^{-1}	IGV	T
As	20	7.23 ± 0.19
Cd	2	0.36 ± 0.007
Co	20	9.96 ± 0.06
Cr	150	56.8 ± 0.61
Cu	120	54.5 ± 0.38
Mn	-	644 ± 3.1
Ni	120	44.0 ± 0.32
Pb	100	14.16 ± 0.12
Zn	150	82.9 ± 3.0

However, at the end of the experiment, significant higher pseudo-total metal concentrations than controls were found for Co, Cr and Cu in AD, together with slightly higher concentration in PS (Fig. 5). For the other elements (As, Cd, Mn, Ni, Pb, and Zn), concentrations in the amended soils were not significantly different from those of the control reported in Table 5.



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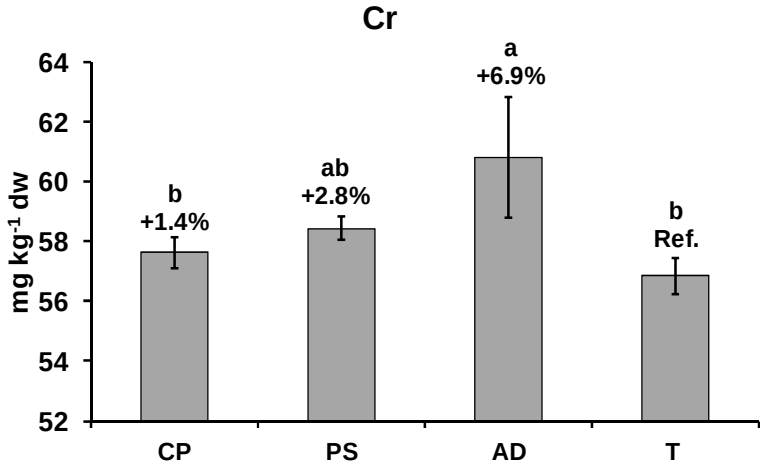

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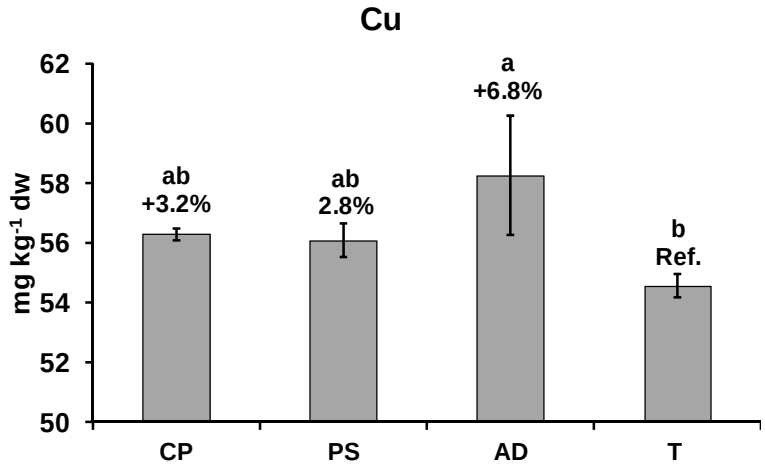
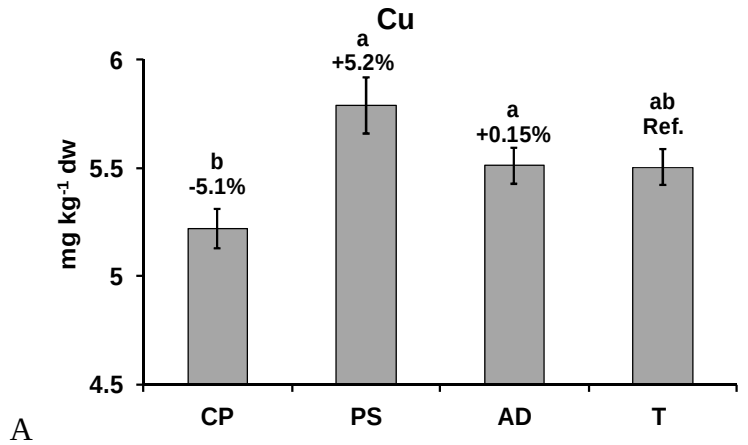
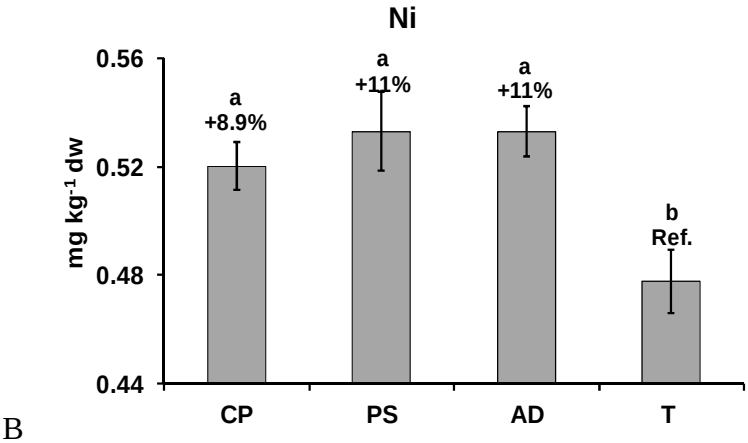


C

Figure 5. Pseudo-total concentration of Co (A), Cr (B) and Cu (C) at end experiment in amended and control soils. Vertical bar represent standard errors. Different letters indicate statistically significant differences among treatments (Student-Newman-Keuls test, $P \leq 0.05$).



A



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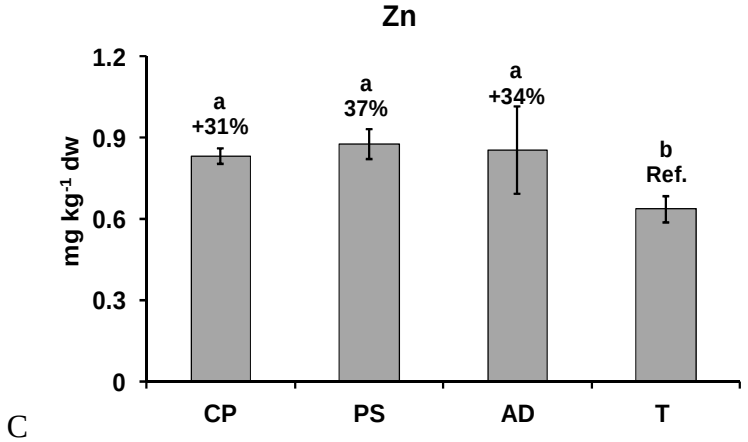
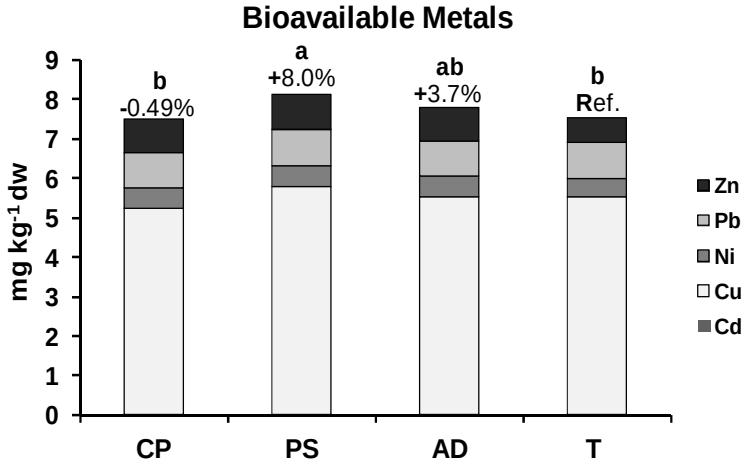


C

Figure 6. Bioavailability of Cu (A), Ni (B), Zn (C) and overall bioavailable metals (Σ(Cd+Cu+Ni+Pb+Zn)) in amended and control soils at harvest. Vertical bars represent standard errors. Different letters indicate statistically significant differences among treatments (Wilcoxon test, $P \leq 0.05$).

Ni and Zn bioavailability generally increased as consequence of organic amendment (Fig. 6B and 6C), and that of Cu was also increased with pig slurry (+5.2%), (Fig. 6A). On the contrary, Cd and Pb bioavailability were very small, 0.01 ± 0.0004 and 0.91 ± 0.02 mg kg^{-1} dw, respectively, and no significant differences were found among treatments.



D

Figure 7. Overall bioavailable metals (Σ(Cd+Cu+Ni+Pb+Zn)) in amended and control soils at harvest. Cadmium is not visible due high figure scale.

The overall bioavailability (sum of metals) had the following order: PS>AD>CP=T, with a significant difference between PS and T only (Fig. 7).

3.3.6. Heavy metal uptake by plant

No statistically significant differences were found among treatments for metal concentrations in the shoots, with similar values at all biomass harvests. On a dry weight basis, average concentrations in the three cuts were 145 ± 23 µg Cd kg^{-1}, 0.011 ± 0.004 mg Co kg^{-1}, 0.55 ± 0.028 mg Cr kg^{-1}, 6.9 ± 0.48 mg Cu kg^{-1}, 62 ± 3.7 mg Mn kg^{-1}, 1.5 ± 0.12 mg Ni kg^{-1}, 0.14 ± 0.015 mg Pb kg^{-1}, 34 ± 3.2 mg Zn kg^{-1}.

3.4. Discussion

3.4.1. Effects of amendments on sorghum

Soil amendment with organic waste materials is efficient in promoting sorghum productivity, confirming the general ability of C-rich organic fertilizers to improve plant growth. This effect was apparently related to N supply (CP>PS>AD>T) at the beginning of the experiment due to amendments, whereas there was no evident relationship with soil P. Indeed, N rate in amendments had a different order, i.e., PS>AD>CP, but the poorer TOC of the more oxidized amendment (CP) forced us to apply a higher amount of compost for reaching similar final C soil contents. On the contrary, none significant correlation was found between root length density (RLD) and soil nutrient contents, although again with compost, together with anaerobic digestate, the maximum growth enhancement was observed. Benefits of pig slurry supply were minimal at above-ground level and negligible at root level, although total soil nitrogen was 20% higher than unamended controls. This result is often detected in fertilized crops, and more frequently in gramineus vs. dycot species, as RLD tends to decrease under elevated nitrogen availability (Bona *et al*., 1995; Vamerali *et al*., 2003b). However, the better root expansion of CP and AD was justified to the hormone-like activity of humic substances extracted from these soils rather than to nutrients availability. According with recent findings of Nardi *et al*. (2009), humic compounds exert various effects on soil properties and, although their plant growth-promoting effects was highlighted in recent years only, there are some commercial products containing humic acids that are claimed to have growth enhancement effects. Plant treatment with biostimulant substances (i.e., lignosulfonate-humate a lignosulfonate-humate b and leonardite) was recently demonstrated to exert a hormone-like activity and be associated with increases in plant biomass (Ertani *et al.*, 2011). In particular, phenolic compounds are thought to be at least in part responsible for the hormone-like activity of humic extracts (Muscolo *et al*., 2007; Pizzeghello *et al*. 2006). Compost and anerobic digestate are composed by mature organic matter due to stabilization processes, oxidization and anaerobic digestion, respectively. During waste treatment there is a significant increase in humification rate (data not shown) and substantial modification of the organic material are possibly responsible for enrichment of

phenolic compounds. In previous study on phytoremediation of pyrite waste, which derives from pyrite ore roasting at very high temperature (~800 °C) and therefore are completely lacking in organic carbon, the addition of a small rate of humic acids (0.1 g kg^{-1} of waste) similarly had a marked root growth enhancement in fodder radish, but not a higher rates (Bandiera *et al*., 2009). In that study it was also evidenced reduced root diameter and improvements of specific root length , a fact that was not found in sorghum, suggesting that the response to HA content might be species-specific.

Besides the general positive effects on plant growth due to organic amendment, no hazardous metal concentration were observed in forage during the whole cultivation cycle, although amendments could seldom increase total and bioavailable soil metals (see following section).

3.4.2. Variations in soil total metal contents and bioavailability

Spreading organic wastes into agricultural lands for supplying organic matter and essential nutrients to crops is often reported to increase total metal contents or bioavailability, rising doubts on possible accumulation of metals in agricultural soils over time and their safe use. For instance, Ramos and López-Acevedo (2004) found that total soil Cu an Ni concentrations were increased after compost addition, and Farrell *et al*., (2010) reported high concentrations of Ni, Cu and Pb and an increase in Cu and Pb leaching after amending soil with municipal solid waste compost.

In this experiment the addition of organic wastes did not resulted in marked increments of heavy metals, even when it appeared significant as in the case of anaerobic digestate and seldom of pig slurry (for Co, Cr, Cu). This suggests that when the selection of organic waste is carried out carefully, metal contents in the amendments are low and the waste-derived amendments might be safely used in agriculture. It is true that in livestock the abundant use of minerals in animal feeding may rise significantly metals in pig slurry, especially of Cu and Zn, but the high DOM of PS and AD at the end of the experiment have probably increased metal mobility and leaching, since the accumulation in plants did not vary significantly with diffrent amendments. Indeed, the increment of metal bioavailability by organic

amendments was generalized, probably because of high concentration of soluble metals in the amendment themselves (Illera *et al*., 2000). In this view, the organic matrixes used here were added at different rates to take into account variations in TOC contents, and this may also have generated changes in metal bioavailability.

The organic compounds of amendments have been often reported in the literature as involved in mechanisms of metal mobility/complexation. In previous studies different concentration patterns were found for heavy metals in the molecular weight (MW) fractions of humic compounds (Francioso *et al.*, 1996; Francioso *et al.*, 2002), but in this experiment the total and bioavailable metal contents in soils is probably independent from the humic compounds content or their MW distribution. As the MW distribution was similar among treatments, it is likely that total bioavailable metals depends on their concentrations in amendments.

A relevant role in metal mobility seems related to the dissolved organic matter, the higher the DOM the higher the bioavailability of heavy metals. High DOM contents are known to facilitate metal mobility because of the formation of soluble metal-complexes, as evidenced by de Zarruk (2007). In particular, here the high Cu bioavailability in the PS treatment was attributed to the formation of soluble Cu-organic compound complexes, since Cu has high affinity for organic matter, and DOM is reported to increase its mobility (Hsu and Lo, 2000). Instead, according to the results of Chirenje and Ma (1999), insoluble high molecular weight organic acids might have retained Cu in the compost-amended soil, thus mainintaining values compareble with unamended controls.

Nickel and Zn increased their bioavailability in the amended thesis irrespective of the source of organic matter. The main variable involved in controlling Zn mobility in soil is pH, with availability sharply decreasing at pH > 5.8 (Yoo and James, 2002). In this experiment pH was alkaline and too high to determine significant metal desorption from soil. Pig slurry had a lower pH compared with compost and digestate and the resulting final soil pH was in any case high. The relatively high Zn availability of all the amended treatments suggests that other mechanisms than pH has contributed to its mobilization, like the high contents of free Zn in the amendments themselves (Smith, 2009). The same conclusion should be drawn for Ni in view of its similar behaviour to Zn.

Among metals, Zn bioavailability was the most increased, a fact also reported by Smith (2009), suggesting that this metal may arise particularl concern for potential leaching from amended soils in long-term, especially if amendments or soils themselves are rich in Zn. Concern in excessive plant accumulation of Zn is currently excluded; on the contrary the concentration of this essential micronutrient was relatively low (~32 mg kg^{-1} dw).

Cadmium and Pb bioavailability were not affected by the addition of organic amendments. In this regards, Cd has low affinity for organic matter, and the adsorption process is independent of organic matter addition (Li *et al*., 2001). The addition of organic materials to the soil can facilitate either Pb stabilization or mobilization (Bradl, 2004), but in calcaric soils, as in this case, precipitation of carbonates is a major mechanism for Pb immobilization (Cao *et al*. 2004). Therefore, it is likely that Pb was immobilized in carbonates and oxides irrespective of amendment presence.

Contrasting results of metal mobility after the addition of organic matter were reported by Walker *et al*., 2003, who found that effects of amendments on metal availability is not related to organic matter composition of the amendments but to soil characteristics. Indeed, results from this experiment on the overall metal availability suggests the opposite conclusion, since different organic matter sources can variously affect metal mobility through variations in DOM and HS rate. It is likely that the same amendment (i.e. compost) can increase or decrease metals bioavailability according to the soil and amendment characteristic itself as suggested by van Herwijnen *et al*., (2007).

3.5. Conclusions

Organic amendments from agro-industrial and municipal wastes can efficiently be recycled in agriculture as they are effective at increasing soil organic matter in the middle term and crop productivity. For equal C stock, it was evidenced that crop productivity is related to nitrogen supply, with possible benefits for root expansion and growth when a considerable amount of humic substance is also supplied with the amendment, as in the case of compost and anaerobic digestate. The

effects on the belowground plant compartment seem very important for ensuring a more efficient use of soil resources (nutrients and water), particularly under stress conditions, such as drought. In this mesocosm experiment, plants were regularly watered but it is likely that in open field conditions plants with larger root systems may have a further advantage. Therefore, the effective restoration of soil fertility in degraded soils necessary requires that amendments provide humic substances along carbon and nutrients.

The addition to the soil of waste-derived amendments does not lead to significant increases in metal concentrations when the amendments are produced from uncontaminated feedstock, and no contraindications in the use of very differentiated organic matrices were found in this study. However, DOM-rich amendments such as pig slurry and anaerobic digestate have higher potential for increasing soil metal mobility, with possible risks of metal leaching with repeated application in agricultural land. The use of such materials should be planned to avoid soil and groundwater contamination in the long-term, especially if hazardous metals like Cd, Pd and Cr are involved.

It was concluded that more stabilized and mature organic amendments, like compost, should be preferred to fresh materials in view of the greater benefits for soil and plants. From the environmental point of view, stabilized composts have also the advantage of avoiding increases of metal bio vailability, providing at the same time more recalcitrant organic matter in the long-term useful for mitigating the greenhouse effects.

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Chapter 4

Long-term effects of biochar on heavy metals in soil and crop plants

Abstract

Biochar incorporation into soil has been advocated as a potential large scale solution to offset global greenhouse gas emissions. However, the application of biochar to agricultural land must have few if any negative economic and environmental consequences if farmers are to readily adopt the technology. Biochar use as an organic amendment has been recently rising due to its positive effect on soil fertility, but there is still limited information available about long-term effects, especially with regard to the effects on soil pollutant content and distribution. In a field-scale trial it was investigated the effect of single doses of biochar (25 and 50 t ha^{-1}) and repeat-applications (in 2009 and 2011) of biochar (25+25 and 50+50 t ha^{-1}) on heavy metal contents (As, Cu, Zn, Cd, Ni) and distribution in soil, along with metal concentration in plants (barley, beans) over repeated cropping cycles. The results indicate that biochar produced from forest residues is of a low risk, due to its inherently low metal content and the lack of observed negative effects on crop or soil quality over several years. Although biochar did cause small changes in metal fractionation in soil, it did not alter total metal concentrations in soil or plants. It was concluded that the application of wood-derived biochar does not increase soil metal contents even after repeated applications deeming it safe for use in agriculture.

4.1. Introduction

Organic soil amendments (e.g. compost, biosolids, manure) are widely used in agriculture to enhance soil nutrient contents and its physical and chemical properties and therefore increasing crop growth [1]. In fact, in addition to stimulating soil microbial activity, soil amendments help conserve soil water, promote nutrient cycling, suppress plant diseases and replenish soil organic matter (SOM) reserves. Although the maintenance of adequate SOM is a major factor for agroecosystem fertility, SOM also contributes to a number of other ecosystem services, e.g. carbon sequestration and waste detoxification [2]. However, poor agricultural management practices have frequently been observed to severely reduce SOM contents, leading to reduced crop yields, chronic declines in soil quality and an increased risk of erosion and desertification [3]. Therefore, there is an urgent need to restore SOM to agricultural soils, and the addition of organic amendments is an important component of all agricultural management regimes. However, due to the progressive biodegradation of organic materials added to soil, their positive effects are typically short-lived and to realize the long-term benefits of SOM there needs to be continual replenishment

Biochar is produced from the pyrolysis of organic materials, and when buried in soil can act as a long term soil carbon (C) store, i.e. remaining for hundreds of years [4, 5]. Burial of biochar in soil has therefore been proposed as a potential mechanism to not only enhance soil fertility, but also to lock up biogenic C, and may play an important role in climate change mitigation by offsetting C emissions associated with the burning of fossil fuels [6, 7]. Many large volume feedstocks are suitable for biochar production, including crop and wood residues, animal manures and a range of industrial wastes such as paper sludge and biosolids [1, 5, 8]. Recent studies have also highlighted the ability of biochar to supply a range of agronomic benefits, e.g. increased nutrient cycling, improved fertility and health [4-6] and enhanced crop productivity, and environmental benefits, e.g. production of bioenergy, global warming mitigation and absorption of heavy metals [4, 6, 9, 10], making it a potentially valuable and sustainable tool to improve soil quality.

Organic amendments are also effective for the remediation of contaminated land, e.g. heavy metal pollution [1], since the rise in both soil pH and cation

exchange capacity (CEC) subsequent to biochar addition can lead to the immobilization of metals through precipitation reactions and adsorption onto organic colloid surfaces [11-13] However, a thorough analysis of the feedstock is needed prior to application to avoid increasing the pollutant load of the soil or the alteration of the mobility/extractability of the indigenous contaminants [14, 15].

Like other organic amendments, biochar can contain high amounts of both organic (e.g. dioxins, polyaromatic hydrocarbons) and inorganic (e.g. heavy metal(loid)s) contaminants, depending on the feedstock and production process [16]. However, there is currently a lack of field-scale experiments providing data about the pollutant content of biochar and the subsequent bioavailability to both crops and soil organisms. This lack of data prevents policymakers from making informed decisions about the risks of amending soil with biochar, together with associated agronomic management decisions and climate change mitigation strategies.

Therefore, the aim of this study was to investigate the influence of variable rates of biochar addition on soil heavy metal concentrations and associated plant uptake in a long-term, field-scale biochar trial within a vegetable-cereal crop rotation system. It was hypothesized that higher biochar addition rates would be more effective at reducing metal availability and plant uptake due to increases in soil pH and CEC and the increased immobilization of metal contaminants. In addition, it was evaluated whether field-aged and fresh biochar had different effects on metal distribution within the soil-plant system.

4.2. Material and Methods

4.2.1. Field experimental set up

The field trial was established in 2009 at Abergwyngregyn, Wales (53°14'N, 4°01'W). The soil is classified as a Eutric Cambisol, has a sandy clay loam texture and is derived from mixed glacial till of Ordovician origin which was deposited approximately 10000 years ago. The replicated (n = 4) trial plots (6 m × 3 m) were laid out in a randomized block design in an existing flat agricultural field that had been used for cereal, vegetable and livestock production over the last 30 years. In 2009, the site was ploughed, harrowed and biochar spread on the surface at rates of either 0 (control), 25 or 50 t ha^{-1}. The biochar was then harrowed into the topsoil (0-20 cm Ah horizon) to ensure mixing. Prior to planting, Reglone® (diquat active ingredient applied at 2 l ha^{-1}) was applied for weed control alongside fertilizer N (100 kg ha^{-1} as NH_4NO_3), P (40 kg ha^{-1}) and K (60 kg ha^{-1}).

The commercially available biochar was derived from mechanically chipped trunks and large branches of *Fraxinus excelsior* L., *Fagus sylvatica* L. and *Quercus robur* L. pyrolyzed at 450 °C for 48 h (BioRegional HomeGrown®; BioRegional Charcoal Company Ltd, Wallington, Surrey, UK). The biochar chip size distribution was 17 ± 1% 0-2 mm, 19 ± 2% 2-5 mm, 32 ± 1% 5-7.5 mm, 32 ± 2% 7.5-10 mm and had a dry bulk density of 0.20 ± 0.01 g cm^{-3}. Further physiochemical details of the biochar, crop and soil management in 2010 and 2011 were provided in previous papers [17-19].

On the 11th June 2011, each of the plots was further split into two 3 × 3 m sub-plots, and biochar of the same origin was then added to half of the sub-plots at rates of 0, 25 or 50 t ha^{-1} to achieve a double loading of biochar and incorporated into the soil as described above. This provided five rates of biochar addition, 0 (control), 25, 50, 25+25 and 50+50 t ha^{-1}. On the 12th July the field was sown (45 seeds m^{-2}) with field bean (*Vicia faba* L. cv. Green Arrow) and Glyphosate (2 l ha^{-1}) and Stomp (active ingredient pendimethalin applied at 3 l ha^{-1}) were applied four weeks later to control weeds. Emergence was completed two weeks after sowing and plants were harvested at 60 days after emergence, along with soil samples for analysis. The results for plants and soil analysis were previously reported [19]. In 2012, spring barley (*Hordeum vulgare* L.) was sown, with no further fertilizer additions.

4.2.2. Soil and biochar analysis

In February 2012, four replicate soil samples (0-20 cm) were taken from each plot and within 1 h of collection soil samples were sieved to pass 5 mm and used for chemical analysis within 24 h. If the soil hadn't been sieved, it would have been introducing a bias into the analyses of the samples containing the high rates of biochar, as the properties of the fresh biochar would have been essentially measured. Instead the aim was to evaluate how the biochar application had affected the soil, and within this, it was analyzed any biochar fragments below 5 mm as part of the total soil sample. The samples were frozen at -20 ^{o}C when not in use. Measurements of basal soil respiration at quasi-steady state were made on 30 g of field-moist soil for 24 h at 20 °C using an automated multichannel SR1 infrared gas analyzer soil respirometer (PP Systems, Hitchin, UK) 24 h after collection from the field. Water content was determined by drying at 105 °C (24 h) and EC and pH were determined with standard electrodes on field-moist soil (1:1 v/w soil-to-distilled water). Available NO_3^- and NH_4^+ were determined in 0.5 M K_2SO_4 extracts (1:5 w/v) using the colorimetric methods [20] and [21] respectively. Cation exchange capacity (CEC) and available nutrients (B, Ca, K, Mg, Na, P, S) were measured at an ISO9001 and ISO17025 accredited laboratory (Lancrop Laboratories, Yara UK Ltd., York, UK). The concentration of arsenic and heavy metals (Cu, Ni, Pb, Zn) in oven-dried soil (105 °C, 24 h) samples collected at the end of September 2011 (and stored at 4 °C) and in February 2012 and fresh biochar were determined by a 700 series ICP-OES (Varian Inc., Palo Alto, CA) after digestion in concentrated HNO_3 [22]. Prior to ICP-OES analysis, all samples were filtered through a nylon 0.45 μm syringe filter.

Sequential extraction of As and heavy metals in soil were also undertaken [23, 24]. Briefly, for the first step (water soluble fraction), 1 g of dry soil or biochar was mixed with 30 ml of distilled water, shaken for 16 h (200 rpm min^{-1}), centrifuged (3000 rpm min^{-1}, 15 min) and filtered (Whatman No. 42). For the second step (surface adsorbed fraction), samples were re-suspended in 30 ml of 0.5 M $NaHCO_3$ and shaken, centrifuged and filtered as described above. For the third step (Fe and Al-associated fraction), the residue from the previous step was re-suspended in 30 ml of 0.1 M NaOH and treated as above. For the fourth step (carbonate bound fraction) the residue from the third step was re-suspended in 30 ml of 1 M HCl and

treated as above. Finally, the residual pellet was dried at 37 °C for 48 h and digested in concentrated HNO_3 to measure residual As and metal contents [22].

4.2.3. Plant analysis

Bean and barley green leaf samples were collected from each sub-plot (ca. 100 g FW) in September 2011 (growth stage R4) and May 2012 (growth stage 31) respectively. The leaves were subsequently dried (80 °C, 48 h), ground (<1 mm), digested in concentrated HNO_3 [25], filtered (0.45 µm) and total As and metal concentrations measured by ICP-OES as described above. In August 2012, the mature barley was harvested and crop height, tiller number and dry seed yield (dried 80 °C, 24 h) measured for each individual sub-plot.

4.2.4. Statistical analysis

All experiments were performed at least in triplicate. After checking for normality and homogeneity of variances, differences in treatments were compared by one-way ANOVA and Tukey HSD (for soil properties) or Duncan post-hoc tests (for heavy metals) (SPSS v.14, SPSS Inc., Chicago, IL).

4.3. Results

4.3.1. Soil and biochar characteristics

Table 1. Influence of biochar application rate on soil quality indicators and available cations. Values represent mean (n = 4) ± standard errors expressed on a dry weight basis. Different letters indicate statistically significant differences among treatments (HSD test, $P \leq 0.05$). The 25 + 25 and 50 + 50 treatments indicate a repeated application of biochar.

	Biochar addition rate (t ha^{-1})				
	0 (Control)	25	50	25 + 25	50 + 50
Basal soil respiration (mg C kg^{-1} h^{-1})	0.47 ± 0.03 (b)	0.46 ± 0.02 (b)	0.50 ± 0.08 (b)	0.53 ± 0.04 (b)	0.71 ± 0.01 (a)
Moisture content (%)	25.1 ± 0.6 (ab)	25.2 ± 0.3 (ab)	24.5 ± 0.4 (b)	27.0 ± 1.2 (ab)	27.4 ± 1.3 (a)
EC (µS cm^{-1})	21.5 ± 1.7 (b)	23.0 ± 0.9 (b)	20.4 ± 1.2 (b)	38.1 ± 0.9 (a)	55.3 ± 4.8 (a)
pH	6.80 ± 0.04 (b)	6.85 ± 0.10 (b)	6.65 ± 0.17 (b)	7.03 ± 0.11 (ab)	7.55 ± 0.03 (a)
CEC (meq kg^{-1})	167 ± 7 (a)	161 ± 5 (a)	122 ± 5 (b)	127 ± 3 (b)	133 ± 3 (b)
B (mg kg^{-1})	0.96 ± 0.06 (b)	1.00 ± 0.06 (b)	0.95 ± 0.05 (b)	1.10 ± 0.02 (b)	1.41 ± 0.04(a)
Ca (mg kg^{-1})	2640 ± 109 (a)	2546 ± 134 (a)	1882 ± 37 (c)	2078 ± 75 (bc)	2372 ± 61(ab)
K (mg kg^{-1})	81 ± 7 (c)	94 ± 4 (bc)	77 ± 6 (c)	131 ± 17 (b)	185 ± 15 (a)
Mg (mg kg^{-1})	62.3 ± 1.6 (b)	71 ± 2.3 (b)	57.3 ± 2.1 (b)	80.0 ± 7.6 (b)	121 ± 10.3 (a)
Na (mg kg^{-1})	29 ± 1 (a)	30 ± 1 (a)	25 ± 1 (bc)	24 ± 1 (c)	23 ± 1 (c)
P (mg kg^{-1})	42 ± 1	42 ± 1	43 ± 2	46 ± 1	44 ± 2
NO_3^- (mg N kg^{-1})	10.6 ±3.1	7.5 ± 4.5	9.1 ± 4.4	5.5 ± 4.3	1.6 ± 0.3
NH_4^+ (mg N kg^{-1})	9.1 ± 0.9	9.2 ± 1.6	10.2 ± 1.1	7.6 ± 0.8	6.9 ± 0.1

There was no significant difference in basal soil respiration between the unamended soil and the soil that had contained the biochar for four years (Table 1). The additional application of 25 t ha^{-1} also made no difference to respiration, however in soil with the highest biochar content rate (50+50 t ha^{-1}), the rate of respiration was significantly higher ($P < 0.05$). The pH and EC remained similar between the unamended control and the soil containing field-aged biochar, while the

highest reapplication of biochar (50+50 t ha^{-1}) significantly increased pH ($P < 0.05$); in addition, both reapplications (25+25 and 50+50 t ha^{-1}) significantly increased EC ($P < 0.05$). The CEC was significantly reduced in the plots containing 50 t ha^{-1} and those containing the reapplications of biochar compared to unamended soil ($P < 0.05$). The concentration of K was significantly higher in soil containing the reapplications of biochar ($P < 0.05$), while B and Mg were only significantly higher ($P < 0.05$) in the soil containing the highest reapplication rate (50+50 t ha^{-1}). In contrast, the concentration of Ca was lower with 50 and 25+25 t ha^{-1} of biochar application (Table 1). There was no significant difference in available P, NO_3^- and NH_4^+ between any of the treatments and the unamended control, regardless of application rate or reapplication.

4.3.2. Total As and heavy metals in soil and biochar

The concentrations of metals in the fresh biochar (1.49 ± 0.22 mg As kg^{-1}; <0.1 mg Cd kg^{-1}; 2.51 ± 0.04 mg Cu kg^{-1}; 1.16 ± 0.08 mg Ni kg^{-1}; 5.98 ± 0.57 mg Pb kg^{-1}; 13.8 ± 2.5 mg Zn kg^{-1}) did not lead to an increase in metal concentration in the soil following biochar addition regardless of the application rate (Table 2). In 2012, small differences in total metal concentration were observed for As, Cu and Ni: slightly higher metal contents relative to the control were found in the 50+50 t ha^{-1} biochar treatment, although the soil containing 50 t ha^{-1} always had significantly lower concentrations than the control. None difference was found for Pb and Zn.

Table 2. Influence of biochar application rate on total soil metal content (mg kg^{-1} dw) as measured in 2011 and 2012. Values represent means ± standard error (*n* = 4). Different superscript letters indicate statistical significant differences among treatments (Duncan test, $P \leq 0.05$).

	Biochar rate (t ha^{-1})				
Metal	0	25	50	25+25	50+50
			Year 2011		
As	11.6 ± 0.4	13.4 ± 1.3	14.3 ± 0.9	12.7 ± 0.8	13.4 ± 0.9
Cd	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.5 ± 0.1	0.1 ± 0.1
Cu	15.1 ± 1.3	17.0 ± 1.3	18.5 ± 1.1	16.4 ± 0.7	18.6 ± 1.3
Ni	12.1 ± 1.3	13.8 ± 1.1	14.6 ± 0.7	13.9 ± 0.4	14.7 ± 0.8
Pb	24.9 ± 0.7	27.2 ± 0.8	29.9 ± 1.5	26.4 ± 2.0	29.3 ± 4.0
Zn	88.1 ± 8.5	92.7 ± 6.0	100.3 ± 4.4	94.0 ± 1.6	103.1 ± 5.1
			Year 2012		
As	9.5 ± 0.6 (ab)	11.1 ± 0.7 (a)	6.6 ± 2.3 (b)	10.0 ± 0.6 (ab)	11.8 ± 1.2 (a)
Cd	0.43 ± 0.05 (ab)	0.45 ± 0.06 (ab)	0.30 ± 0.10 (b)	0.45 ± 0.05 (ab)	0.58 ± 0.07 (a)
Cu	11.2 ± 1.2 (ab)	12.1 ± 1.4 (ab)	8.3 ± 2.0 (b)	11.2 ± 0.9 (ab)	13.6 ± 1.0 (a)
Ni	8.8 ± 0.7 (ab)	9.4 ± 1.3 (ab)	6.2 ± 1.6 (b)	8.7 ± 0.7 (ab)	10.0 ± 1.0 (a)
Pb	19.5 ± 2.3	19.9 ± 1.0	15.9 ± 4.4	20.3 ± 1.1	22.2 ± 1.5
Zn	65.1 ± 7.2	67.1 ± 5.6	49.1 ± 13.7	64.6 ± 5.2	76.1 ± 6.5

4.3.3. Sequential extraction of As and heavy metals

Control soil

The concentration of As and metals in the unamended control soil determined by sequential extraction are reported in Table 3. The concentration of arsenic was evenly distributed between the Fe-associated and Al-associated (step 3) and residual (step 5) fractions, which together constituted 68.0% of the total extracted. The water soluble (step 1), surface adsorbed (step 2) and carbonate-bound (step 4) fraction represented 2.7, 8.9 and 20.0 % of the total As extracted respectively.

Table 3. Amount of arsenic and heavy metals in the reference agricultural soil (0 t ha^{-1} biochar) as found through sequential chemical fractionation (step 1: water soluble; step 2: $NaHCO_3$-extractable; step 3; NaOH-extractable; step 4: HCl-extractable; step 5: residual fraction, HNO_3-extractable). Values represent means ± standard error (*n* = 4). Values in bold represent the maximum rate within each metal.

	Metal concentration in fraction (mg kg^{-1} dw)					
Metal	Step1	Step2	Step3	Step4	Step5	Total
As	0.29 ± 0.13	0.97 ± 0.09	**3.92 ± 0.73**	2.17 ± 0.13	3.50 ± 0.17	10.86 ± 0.85
Cd	<0.01	<0.01	<0.01	**0.46 ± 0.02**	0.17 ± 0.02	0.63 ± 0.02
Cu	0.60 ± 0.19	0.91 ± 0.07	4.04 ± 0.75	**8.47 ± 0.37**	2.95 ± 0.16	16.98 ± 0.75
Ni	0.15 ± 0.04	0.08 ± 0.02	0.71 ± 0.13	5.85 ± 0.19	**6.28 ± 0.34**	13.08 ± 0.24
Pb	0.38 ± 0.09	0.06 ± 0.03	0.55 ± 0.12	**33.6 ± 1.7**	0.85 ± 0.32	35.47 ± 1.66
Zn	3.69 ± 1.24	2.03 ± 0.46	1.48 ± 0.41	**42.2 ± 2.3**	29.9 ± 1.7	79.30 ± 3.57

Cd was extractable only through HCl and HNO_3 (step 4 and 5 respectively), with 27.5% in the residual fraction and 72.5% of the Cd being HCl-extractable.

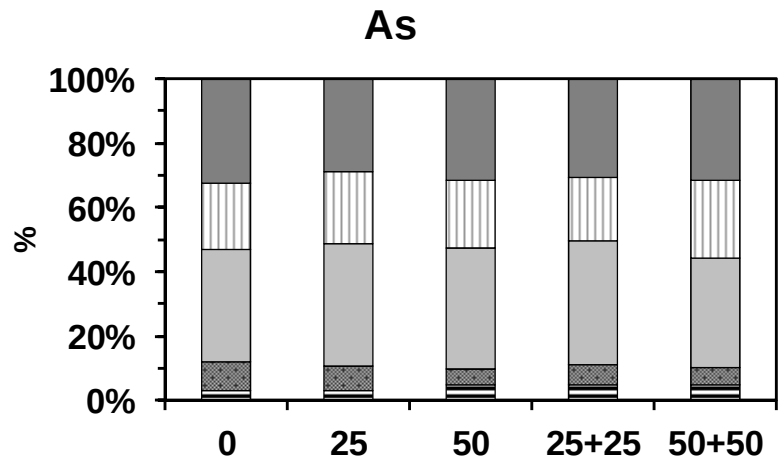
Cu was mainly extracted with HCl (49.9%), however, significant amounts were also found in the NaOH (23.8%) and residual (17.4%) fractions. Smaller amounts of Cu were water soluble and $NaHCO_3$-extractable (3.5 and 5.4% of the total respectively).

The concentration of Ni was evenly distributed among the HCl-extractable and residual fractions (44.7 and 48.0% respectively), while only small amounts (1.1, 0.6 and 5.6%) were water soluble (step 1), $NaHCO_3$-extractable (step 2) and NaOH-extractable (step 3) respectively.

Pb was mostly extracted with HCl (94.8%), and the proportions in the other fractions were therefore very low.

Zn was mainly extracted during the steps 4 and 5 (53.2 and 37.7% respectively). Significant amounts were also found in the water soluble (4.7%) and $NaHCO_3$-extractable (2.6%) fraction, with little Zn being NaOH-extractable.

Soil with biochar



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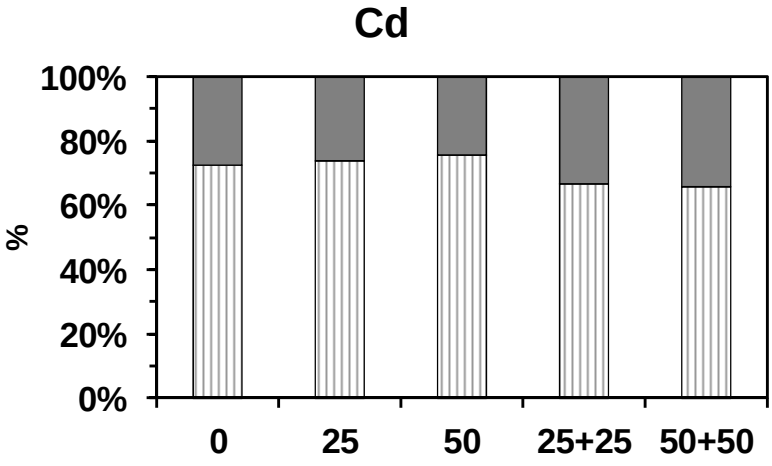

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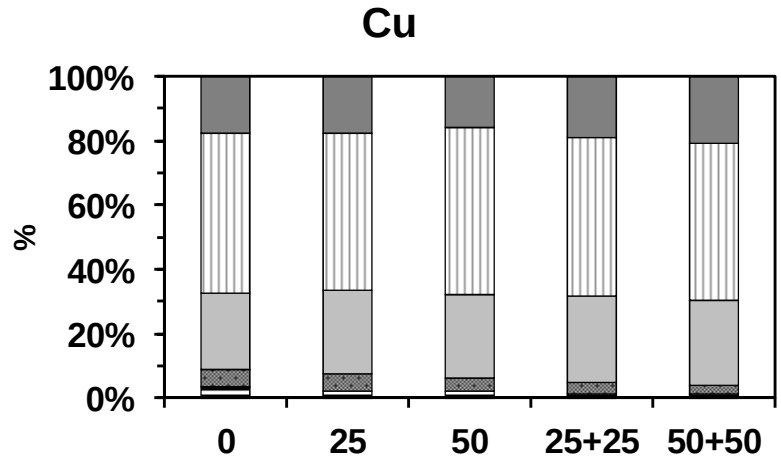

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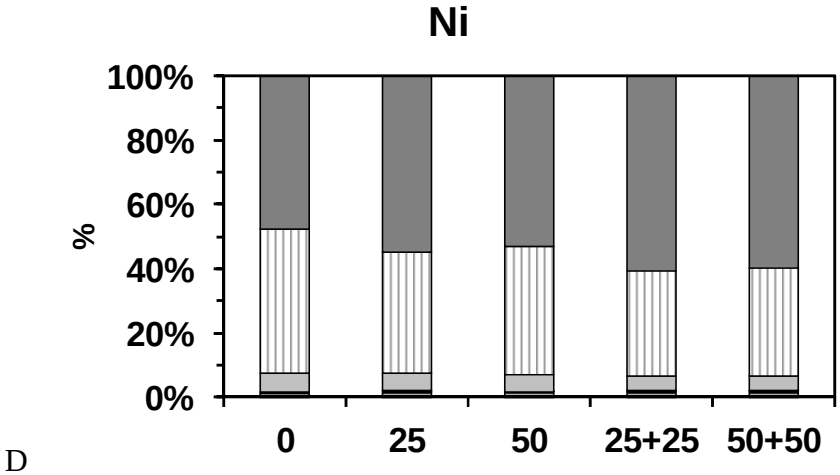

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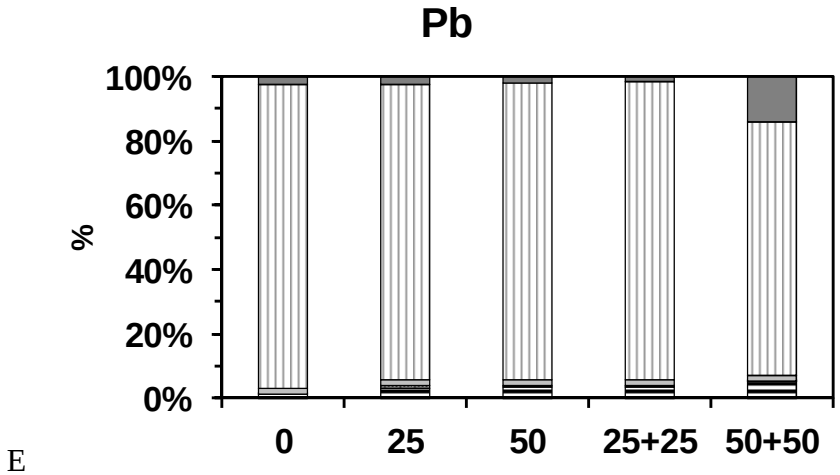

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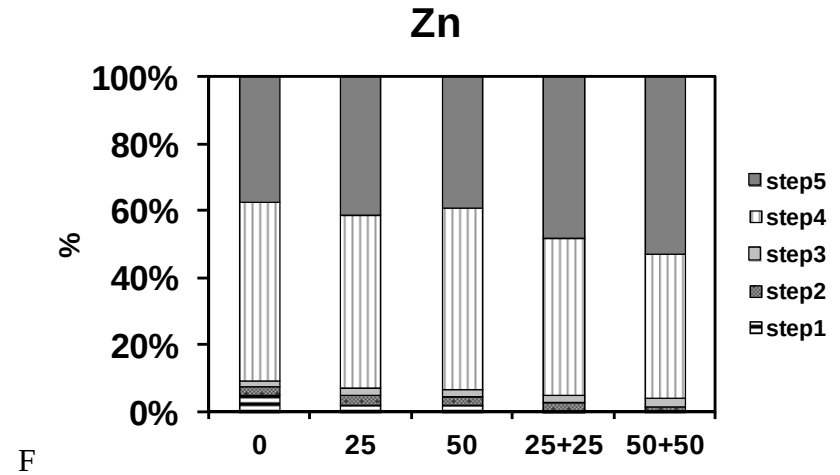


F

Figure 1. Influence of biochar addition rate on the relative distribution of arsenic and heavy metals in the agricultural soil as found through sequential chemical fractionation (step 1: water soluble; step 2: $NaHCO_3$-extractable; step 3; NaOH-extractable; step 4: HCl-extractable; step 5: residual fraction, HNO_3-extractable).

Biochar addition affected both the concentration and relative proportion of As and heavy metals present during the sequential extractions (Fig. 1). Biochar always significantly decreased surface adsorbed As, and increased the amount of As bound to carbonates in the repeated additions. On the contrary, biochar did not affect the total, water soluble, Fe and Al-associated and residual extractability of As.

The total amount of Cd extracted (mg kg^{-1}) was significantly increased in the presence of biochar, especially for the repeat treatments (on average +20.0%). In addition, the repeat application treatments increased the proportion of Cd extractable from the residual fraction (on average +22.1%) and reduced that associated with the HCl-extractable fraction (on average by -8.5%) with statistically significant differences ($P<0.05$) from the control. In contrast, the single-dose treatments seemed to cause the opposite effect (-5.4 and -11.4% in the residual fraction and +2.0 and +4.3% in the HCl-extractable fraction with 25 and 50 t ha^{-1} biochar respectively), even though the difference from the control was only slightly statistically significant.

Water soluble and $NaHCO_3$-extractable Cu concentrations and percentages were reduced at increasing biochar addition rates, with significant differences between single dose and repeat-application treatments. The repeat biochar treatments also significantly increased the amount of Cu (mg kg^{-1}) and proportion in the 5^{th} step. The treatment with 25 t ha^{-1} biochar had no effect on Cu extractability and distribution.

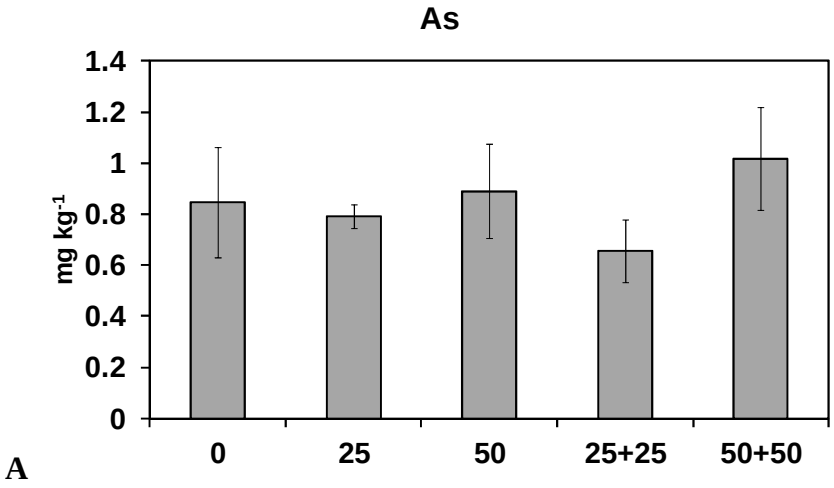
Ni total, water soluble and residual extractability significantly increased with increasing biochar addition rates, with statistically significant differences between single-dose and repeat biochar treatments. In contrast, Ni proportion was significantly reduced in the HCl-extractable fraction (-13.1% and -25.8% for the single and repeated treatments respectively).

Total extractable Pb did not significantly increase with biochar application rate. Nevertheless, water soluble Pb was greatly increased in the presence of biochar, especially at the highest addition rate (+473% with 50+50 t ha^{-1} biochar). No significant differences in Pb were found in the other soil fractions.

Zn total extractability increased on average by 16.0% with biochar; nevertheless water soluble Zn decreased with increasing biochar. In addition, the repeat-biochar treatments increased residual Zn (+42.7 and 75.0% with 25+25 and 50+50 t ha^{-1} biochar respectively) and reduced the HCl-extractable proportion (-11.6

and -18.7%). The same effects were also observed in the single dose treatments but to a lesser extent.

4.3.4. Heavy metal uptake by plants and crop yields



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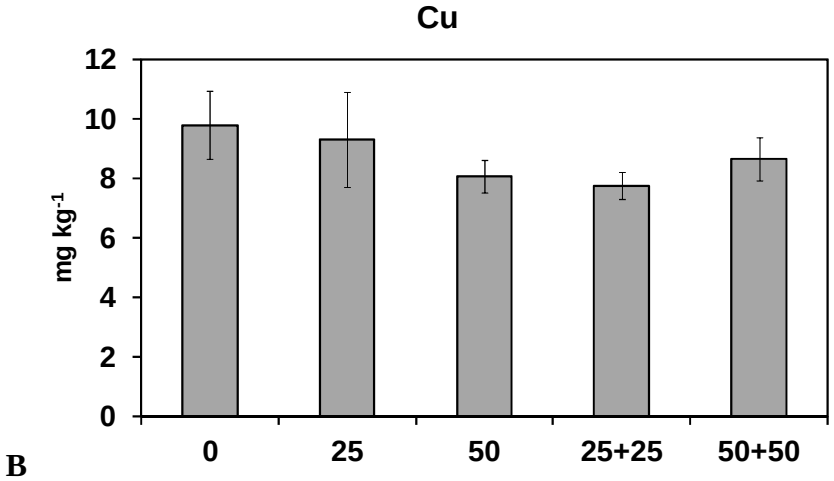

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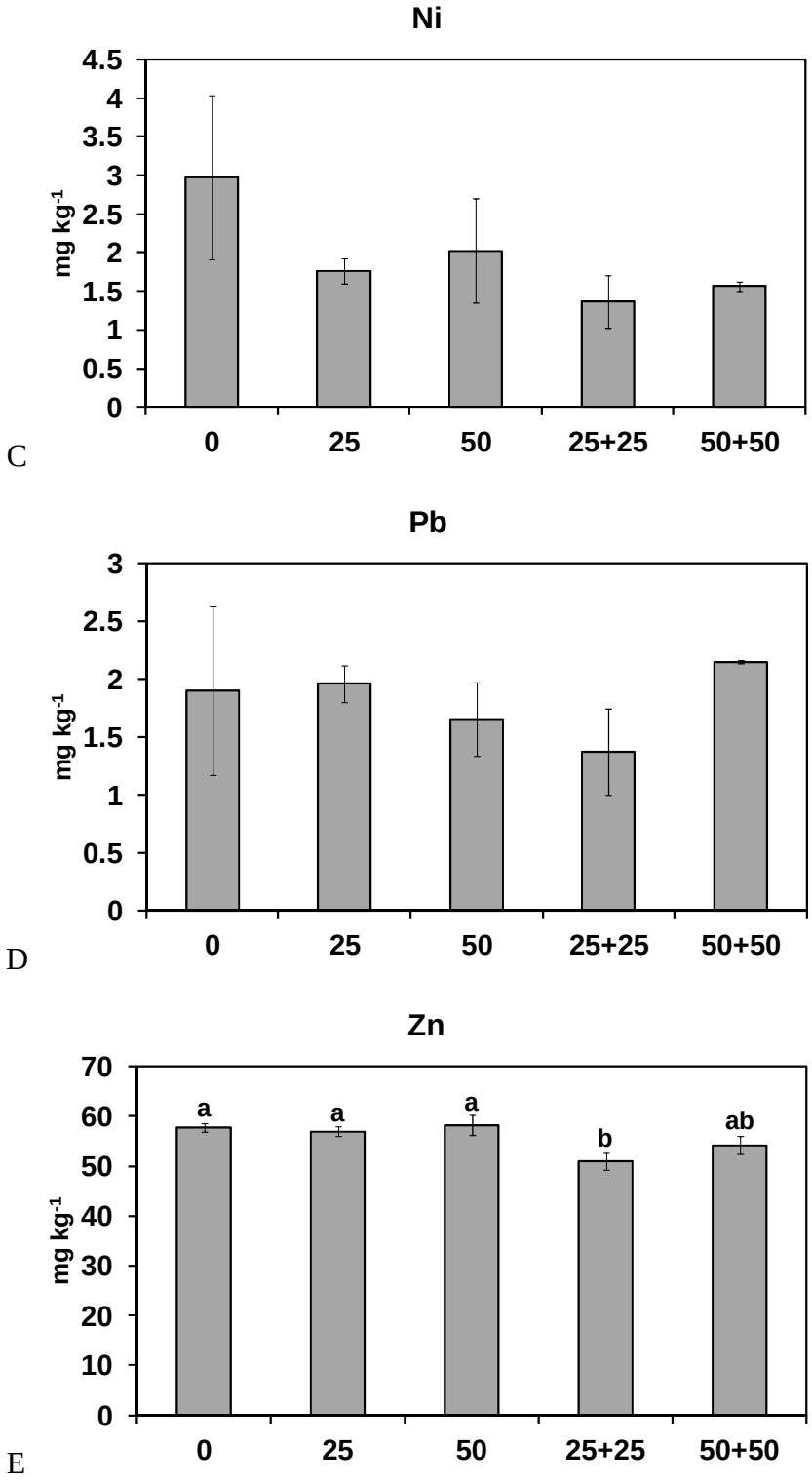

Figure 2. Influence of biochar addition rate on total As and heavy metal content in bean leaves. Values are expressed on a dry weight basis and represent means (*n* = 4). The vertical bars represent standard errors. Different letters indicate statistically significant differences among treatments (Duncan test, $P \leq 0.05$).

Heavy metal concentrations in crop foliage are reported in Figure 2 for beans and Figure 3 for barley. Neither bean nor barley accumulated high amounts of metals, and no significant differences were found among treatments, except for Zn in beans. Cd was not detectable in either crop, while in beans As and Pb were found at very low concentrations (on average 0.8 and 1.8 mg kg^{-1} respectively). In barley only Cu and Zn reached detectable concentrations, with no significant differences apparent between biochar and control treatments.

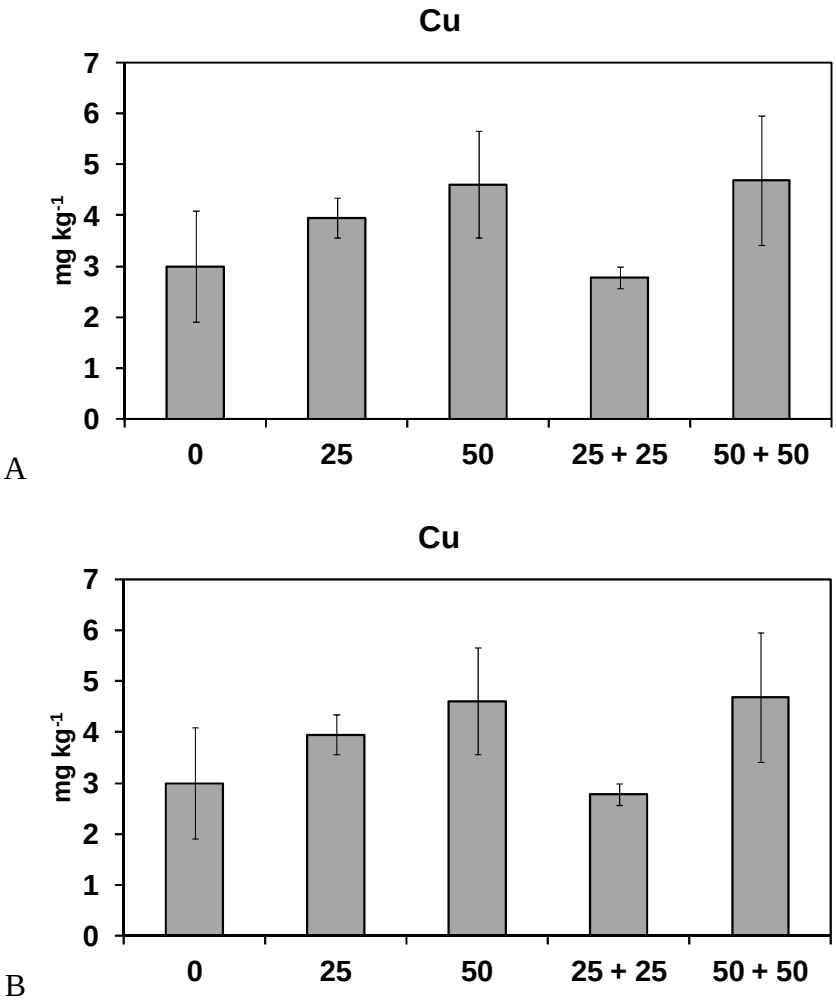

Figure 3. Influence of biochar addition rate on total heavy metal content in barley leaves. Values represent means (*n* = 4). Vertical bars represent standard errors. Different letters indicate statistically significant differences among treatments (Duncan test, $P \leq 0.05$).

At the final harvest, no significant differences in barley yield (P = 0.91), crop height (P = 0.63) or tiller number per plant (P = 0.19) were observed between any of the biochar treatments and the unamended control treatment. The mean barley grain yield across all treatments was 7.2 ± 0.1 t ha^{-1}.

4.4. Discussion

4.4.1. Effects of biochar application on soil quality

This study suggests that the addition of biochar to agricultural soil directly influences both micro and macro nutrient solubility. However, nutrient enhancement was only primarily apparent in the repeat-biochar treatments, therefore nutrient benefits are likely relatively short lived [19]. In contrast, the loss of exchangeable Ca and a lowering of the CEC appear to be negative consequences of the high rates of fresh biochar addition.

Although aging can cause significant changes to the surface properties of biochar (e.g. decreasing aromaticity and the formation of carboxylic groups) [10], in the soil containing field-aged biochar there was generally no alteration of soil properties compared to unamended control.

Biochar addition also had a liming effect on soil pH, probably due to dissolution of metal hydroxides and carbonates present in the fresh biochar (e.g. CaO, $CaCO_3$) [17], although this too appeared to be short-lived.

4.4.2. Effects biochar on the proportions of extractable As and heavy metals

The extraction method used in this study was developed specifically for As fractionation [23], and it is likely that heavy metals were extracted from different phases than As. In addition, it is likely that HCl extracted metals can bind to organic matter [24], and metals extracted from both Fe and Mn oxides and residual minerals could underestimate the percentage of the residual fraction. The residual fraction often comprises the largest amount of metals [26], although here it was often found a

relatively small percentage of metals in the last extraction step, e.g. Cu, Cd and Pb. However, as the extraction was primarily aim at measuring the water soluble and HCl-extractable fractions, the results remain important for the risk assessment of soil contamination by heavy metals following biochar addition.

The application of biochar significantly altered the extractability of both As and heavy metals in soil, with biochar shifting the extractability of some metals (As, Cu, Zn) from the water soluble and exchangeable fractions to the HCl-extractable and residual fractions or increasing the water soluble proportion (Pb). Shifts in cation exchange and changes in pH are recognized as the main drivers of metal mobility and sorption following biochar addition [27, 28]. However, the results suggest that other mechanisms might be involved in biochar-metal interactions. There were significant reductions in the water soluble fractions (Cu, Zn), despite generally lower CEC values in soil containing field-aged biochar compared to the control. Biochar age also seems to be involved with determining the fate of metals in soil despite field-aged soil having a similar pH to the control. Recent studies suggest that SOM can influence the formation of As(III) in aerobic environments by mediating the reduction of soluble As(V) to less soluble As(III) [29]. Therefore, it is likely that higher rates of organic matter addition can enhance As retention. The arsenate absorption on humic acid has a peak at pH = 7 [30], and the reduction in exchangeable As after biochar addition could be due to insoluble As-biochar complexes, since biochar sorption behavior is similar to that of SOM [31].

The concentration of Cd in the HCl-extractable and the residual fraction was dependent on both the application and reapplication rate of biochar. Cd is mainly found in the exchangeable fraction [32, 33], although other soil phases (i.e. Fe and Mn oxides, organic carbon, water soluble) can contain significant amounts of this metal [24, 34-36,]. The absence of Cd in the water soluble fraction in this study may have been due to the very low Cd content in both soil and biochar, while its absence in step 2 and 3 of the extraction was likely due to the high pH of the extraction solutions. It is possible that different insoluble Cd-organic complexes were formed as a consequence of Cd being more effectively retained by aromatic rings rather than carboxylic and phenolic groups. Consequently, its retention should be enhanced following the addition of fresh biochar, while the field-aged biochar would result in a shift from the residual fraction towards the HCl-extractable fraction (less insoluble).

Oxidation of biochar can lead to the formation of carboxylic and phenolic groups at the expense of aromatic rings [10], therefore, different biochar feedstocks could result in very different retention ability and interactions with different heavy metals.

Cu has a stronger affinity for SOM than other heavy metals [37-39] and its availability is reduced by organic amendments [14] and biochar addition [31, 40] due to effective absorption mechanisms. The results find here are consistent with these observations, as increasing biochar addition rates significantly reduced the concentration of water soluble and $NaHCO_3^-$extractable Cu. Moreover, it is likely that the higher pH enhanced Cu retention in the repeat-biochar treatments, thus explaining the lower water soluble amount found with 25+25 and 50+50 t ha^{-1} biochar applications compared to the other treatments. Similarly, Ni retention has recently been found to be enhanced by biochar [28, 41], probably as a consequence of reduced mobility at the higher pH. The highest concentration of water soluble Ni was found in the soil that contained the reapplications of biochar (25+25 and 50+50 t ha^{-1}) that had the higher pH values. However, the increase in the proportion of water soluble Ni with increasing biochar application rates was probably due to the higher Ni concentration of the biochar, as indicated by the increase in total extractable Ni from biochar amended soil, rather than to a higher solubility due to the addition of biochar.

In contrast to previous studies [9, 40], it was found that the amount of water-soluble Pb increases following biochar addition suggesting that biochar might have different effects on Pb mobility according to soil conditions. Precipitation of insoluble Pb-phosphate minerals can determine Pb solubility [42], and the low percentage of labile Pb was probably a consequence of the high P concentration in all treatments. The increase in the concentration of water soluble Pb may also have been related to the formation of soluble Pb species, such as Pb-nitrates [31], especially in the repeat-biochar treatments, where the nitrate concentration decreased with the higher biochar application rates. However, the possibility that competition with Cu and Zn for sorption sites may have resulted in the higher Pb solubility can not be excluded.

Reduction in Zn extractability and increased retention following biochar addition is well documented [43-45]. The altered proportions of extractable Zn in soil suggests that Zn is shifted from the most soluble to the most insoluble fractions as a

consequence of biochar addition. Total Zn extractability was significantly increased in soil with reapplications of biochar (25+25 and 50+50 t ha^{-1}) compared to the control, although this effect could be due to the relatively high amount of Zn in biochar [31].

4.4.3. Effects of biochar on plant uptake of heavy metals

Biochar has previously been reported to reduce metal bioavailability in contaminated soils [31, 46, 47], which in an agricultural context could potentially lock up metals, inducing micronutrient deficiencies and reducing yields. This could arise indirectly by a rise in soil pH reducing metal solubility or directly via metal binding to biochar surfaces. the results found here, however, show that the biochar-induced increases in soil pH are transient, and that biochar amendment of this particular agricultural soil reduces the CEC. Further, it was found that biochar did not significantly affect foliar micronutrient content or crop yields, and where an increase in the water soluble metal fraction was observed in response to biochar addition (e.g. Ni and Pb), no concomitant increase in foliar concentration was observed. These findings support reports that suggest that biochar will have few short or long-term deleterious effects on plant growth.

4.4.4. Biochar metal content and the relevance to agriculture

Currently, there is no consensus on the effects of biochar on soil metal availability [48-50], which is hampering the formulation of guidelines for the safe application of pyrolysis products to land. The impact on soil metals will be controlled to a large extent by feedstock quality, with chars derived from anthropogenic wastes (e.g. biosolids) likely to induce high metal loadings and elevated environmental risk in comparison to chars derived from more natural products (e.g. animal manures, forest residues). However, initially there is need to get public and farmer acceptance of those chars perceived to be of low risk before advocating the application of industrial waste-derived chars.

Here a low metal-content char was used, which was produced from a high volume feedstock that is capable of meeting the needs of agriculture, if biochar is to be seen as a significant component of any national greenhouse gas reduction program. Overall, the commercial biochar used here possessed a low heavy metal burden in comparison to other waste streams routinely applied to land (e.g. tannery waste, biosolids, municipal solid waste composts) [13]. These findings also concur with biochars produced from wood in laboratory scale reactors [51, 52].

The combination of soils and biochar with low metal content represents the scenario most likely to occur in temperate agricultural soils across Europe. This contrasts with many previous studies which have focused on metal contaminated soils or chars and which have limited current relevance for widescale technology or policy adoption. Importantly, the results obtained here confirm that repeat application of low risk biochar does not appear to lead to a progressive increase in soil metal load, suggesting that any excess metals are either taken up by plants, redistributed in the soil profile (subsoil transfer) or lost by leaching. Previous research has indicated that our biochar contains large amounts of dissolved organic C (DOC) [18], which is readily released upon application to soil. It is therefore probable that this facilitates the downward movement of metals within the first few months of biochar application, but groundwater contamination due to the low metal concentrations in both soil and biochar was excluded. However, clearly more work is required to confirm this pathway.

4.5. Conclusions

Biochar incorporation into soil has been advocated as a potential large scale solution to offset global greenhouse gas emissions. However, the application of biochar to agricultural land must have few if any negative economic and environmental consequences if farmers are to readily and safely adopt the technology. Here it was found that biochar produced from forest residues is of a low risk due to its inherently low metal content and the lack of observed negative effects on crop or soil quality over several cropping cycles. It was therefore concluded that wood biochar application does not increase soil metal contents even after repeated

applications, although attention should be paid to the quality of pyrolized material. Repeated application of biochar in agricultural soils should be considered with caution for some soil properties only, like CEC, worsening of which has been observed at a cumulative rate of 100 t ha^{-1}.

Acknowledgements

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4.6. References

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Chapter 5

Waste wood-derived biochar and ash increase the bioavailability of metals in two contrasting agricultural soils

Abstract

Recycled waste wood is being increasingly used for energy production, however, organic and metal contaminants in by-products produced from the combustion/pyrolysis residue may pose a significant environmental risk if they are disposed of to land. Here it was conducted a study to evaluate if biochar (from pyrolysis) and ash (from incineration) derived from metal-preservative treated wood led to significant accumulation of metals (e.g. Cu, As, Ni, Cd, Pb, Zn) in soil and vegetation. In a pot experiment, biochar (2% w/w), corresponding to 50 t ha^{-1} and an equivalent pre-combustion dose of wood ash (0.2 % w/w) were added to a Eutric Cambisol and Haplic Podzol, respectively. Both amendments initially raised soil pH, however, this effect appeared relatively short term with soil pH returning close to the unamended control within about 7 weeks. While both amendments significantly increased the bioavailability of plant nutrients (e.g. K), their addition resulted in an exceedance of soil metal statutory limits (e.g. Cu). The metal sorbing capacity of the biochar and the increase in soil pH caused by adding the ash and char were insufficient to offset the amount of free metal released into solution. Young sunflower plants were negatively affected by the addition of metal treated wood-derived biochar and resulted in an elevated concentration of metals in the tissues, especially of roots, and reduced above- and below-ground biomass. It was concluded that biochar and ash produced from waste streams containing metal based preservatives should not be used as a soil amendment due to the high risk of environmental contamination, in this case of Cu.

5.1. Introduction

Organic wastes are of global concern due to the large volumes produced and the need to dispose of them safely (Jones and Healey, 2010). Organic waste has traditionally been disposed of through incineration or landfill, but recently there has been a worldwide tendency to recycling and adopting a more sustainable waste management program (Vehlow *et al*., 2007), with priority to preventing and reducing biogenic waste (Del Borghi *et al*., 2009). As a result, the most widely adopted strategy for the recycling of organic waste is to incorporate it into agricultural soils after treatment that turn the waste into safe and efficient amendments (e.g. via composting or anaerobic digestion; Park *et al*., 2011a; Williams, 2005). This practice, in addition to reducing unnecessary landfill, can replenish soil organic matter reserves, provide plant nutrients and help close the nutrient cycling loop (Jones and Healey, 2010). There are numerous reports demonstrating the positive effects of adding municipal- and industrially-derived organic wastes to land (Linden *et al*., 1995; Curnoe *et al*., 2006; Hargreaves *et al*., 2008).

In addition to being used as compost, organic wastes (e.g. recycled waste wood, biosolids) can be used for energy generation via pyrolysis or incineration, which results in the production of biochar and ash respectively (Campbell, 1990; Lehmann, 2007). These end-products can then provide further benefit by addition to agricultural soils as an organic amendment or liming agent (Demeyer *et al*., 2001; Atkinson *et al*., 2010). Although biochar application to temperate agricultural soils can transiently increase the concentration of nutrients such as P, K and Ca (Quilliam *et al*., 2012a), its real value lies in providing a long-term recalcitrant store of carbon in soil (Kookana *et al*., 2011). In contrast, wood ash is mainly used to raise the pH of acidic soils, although it can also provide a significant source of nutrients, particularly Ca, K, Mg and P (Someshwar, 1996), with significant benefit to crop productivity (Patterson *et al*., 2004).

An inherent risk of applying biochar and wood ash to soil is that they contain varying levels of contaminants including those generated during pyrolysis (e.g. polyaromatic hydrocarbons (PAHs), dioxins and furans (Hale *et al*., 2012; Quilliam *et al*., 2012b) and, depending on the feedstock (treated wood or municipal biosolids), both biochar and wood ash can contain significant concentrations of heavy metals.

PAHs have been found at measurable concentrations in a number of different source-derived synthetic and natural chars and ash, and often exceed regulatory standards (Reijnders, 2005; Brown *et al*., 2006; Hale *et al*., 2012). Biochar can reduce microbial catabolism of PAHs in soil through increased sorption and reduced bioavailability which, together with elevated concentrations in soils amended with biochar, can facilitate the persistence of PAHs in the environment (Quilliam *et al*., 2012b). In general, there appear to be few major impacts on heavy metal behavior following the addition of wood-derived biochar to soil; however, biochar has the capacity to both sorb and release metals (Namgay *et al*., 2010; Uchimiya *et al*., 2010a,b). In contrast, wood ash addition can increase trace element concentrations in soil and plant tissues and stimulate metal leaching (Omil *et al*., 2007; Praharaj *et al*., 2002), although a reliable evaluation of the leaching from wood ash is difficult to assess, since it varies depending on both the ash feedstock and the composition of the leaching solution used (Solo-Gabriele *et al*., 2002). This makes it difficult to compare results and establish the potential risk for contamination, and consequently there are currently no guidelines for the safe and effective use of soil amendments such as waste-derived biochar and wood ash.

The aim of this study was therefore to determine whether amendment of agricultural soils with biochar and ash derived from preservative-treated wood increases the metal concentration in the soil and negatively affects plant growth. It was hypothesized that total metal concentrations in the soil would increase following amendment with both biochar and ash, although this would not be accompanied by higher levels of plant uptake as the effective adsorption onto biochar and wood ash surfaces, together with an increase in pH, would decrease the bioavailability of metals (Su and Wong, 2004; Chirenje *et al*., 2006; Namgay *et al*., 2010).

5.2. Material and Methods

5.2.1. Biochar and wood ash production

Biochar and wood ash were produced from Norway Spruce (*Picea abies* (L.) H. Karst.) waste wood, which had previously been pressure-treated with a Cu-based wood preservative. The wood (35 × 120 × 2500 mm) had previously been used outdoors in North Wales, UK (mean annual temp 11 °C; 1800 sunshine h y^{-1}; 840 mm rain y^{-1}) for 4 years prior to disposal and reclamation. Biochar or ash were produced by pyrolysis or combustion respectively, at 550 °C for 1 h and then left to cool for 24 h. Biochar was ground and sieved to pass 5 mm before use. The ash was in a powder form following combustion and did not require grinding.

5.2.2. Experimental design

Two contrasting soils, a sandy clay loam textured Eutric Cambisol (78 g kg^{-1} organic matter) and a sandy loam textured Haplic Podzol (509 g kg^{-1} organic matter), were collected from the University Experimental Station at Abergwyngregyn, Wales (53°14'N, 4°01'W). The freely draining Eutric Cambisol supports a sheep-grazed (ca. 10 ewes ha^{-1}) grassland sward dominated by *Lolium perenne* L. and *Trifolium repens* L., and receives regular fertilizer applications (120 kg N ha^{-1} y^{-1}). The freely draining Haplic Podzol supports a sheep-grazed (ca. 0.1 ewe ha^{-1}) grassland sward dominated by *Festuca ovina* L. and *Pteridium aquilinum* (L.) Kuhn, and commonly receives no fertilizer or lime. Soil was sampled from 0-30 cm depth and sieved to pass 5 mm before use. Further details of the soils and their physiochemical properties are presented in Table 2 and Farrell *et al*. (2011).

To reflect typical land management practices and application rates in the UK, biochar was added to the Eutric Cambisol at a rate equivalent to 50 t ha^{-1} (i.e. as a C sequestration agent; Sohi *et al*., 2010; Jones *et al*., 2012) corresponding to 2% w/w, while the wood ash was added to the Haplic Podzol at a rate of 5 t ha^{-1} (i.e. as a liming agent; Pitman, 2006) corresponding to 0.2% w/w. The high ash content of the biochar (10% w/w), made these application rates directly comparable, albeit the responses were in different soil types. Wood ash was not added to the Eutric

Cambisol as this was predicted to cause excessive alkalinization of the soil while biochar had no effect on pH or plant growth in the Haplic Podzol during preliminary trials.

Black plastic pots (1000 cm^3) were filled with field-moist soil from each of the four treatments: (1) Eutric Cambisol, (2) Eutric Cambisol + biochar, (3) Haplic Podzol, (4) Haplic Podzol + wood ash. The replicate pots (n = 5) were then transferred to a glasshouse (20 ± 2 °C with natural daylight) and left to equilibrate for 7 d. Four sunflower seeds (*Helianthus annuus* L. cv. Sunburst) were sown in each pot, which received every day up to 100 ml of distilled water. A commercial fertilizer (MiracleGro®, NPK 24-8-16; Great Garden Supply, Boston, MA) was applied (50 kg of N on a hectare basis) at 14 d after sowing. At 21 d after sowing, seedlings were thinned to two per pot, and at 45 d after sowing all plants were harvested.

5.2.3. Substrate analysis

Biochar and wood ash pH was determined with standard electrodes (1:10 w/v in distilled water). Total As, heavy metals (Cd, Cu, Ni, Pb and Zn) and cation (Na, K, Ca) contents of the ash and biochar were determined by a Agilent 700 Series ICP-OES (Varian Inc., Palo Alto, CA) after digestion in concentrated HNO_3 and filtration through nylon 0.45 µm syringe filters according to US-EPA (1995a).

Soil pH and EC were determined on field-moist soil (1:1 w/v soil-to-distilled water) with standard electrodes at the beginning and end of the experiment. Moisture content was determined by drying at 105 °C for 24 h. Exchangeable cations (K, Na and Ca) were extracted in 1:5 (w/v) fresh soil suspension using 1 M NH_4OAc (pH = 7) after shaking at 250 rpm for 1 h (Helmke and Sparks, 1996), and analyzed by flame photometry (410 Flame Photometer; Sherwood Scientific, Cambridge UK). Soil As and total heavy metal contents were determined by ICP-OES as described above. Nutrients and metal bioavailability was measured according to Lambrechts *et al*. (2010) at beginning and end of the experiment; briefly, 25 ml of 0.01 M $CaCl_2$ was added to 2.5 g of air-dried soil, shaken for 24 h, centrifuged (3000 *g*, 15 min), filtered through successive Whatman 42 filter papers and 0.45 µm nylon syringe

filters and analyzed by ICP-OES. The extraction solution was left unbuffered to avoid altering metal speciation and solubility in soil (Houba *et al*., 2000, Meers *et al*., 2007). Arsenic bioavailability was measured with the same method, since As concentration in shoot and root is well correlated to the $CaCl_2$-extractable fraction (Vázquez *et al*., 2008).

5.2.4. Metal speciation modelling

The amount of free metal (i.e. uncomplexed) in soil solution in response to the addition of metal contaminated wood ash or biochar was predicted using the chemical speciation program Geochem-EZ for Windows v1.0 (Shaff *et al*., 2010). The initial soil metal loading rates were taken from Table 1 (Eutric Cambisol + biochar and Haplic Podzol + wood ash) and the model was run over the range of fixed pH values observed in the experiments. Solids were allowed to precipitate within the model runs, which typically took between 2 and 7 iterations to achieve convergence. P and S contents of the biochar/ash were also included in the model and the input data used were based on Barrelet *et al*. (2006) although it was assumed that all S was lost during wood ash formation. Cl^- and NO_3^- were assumed to balance any excess cationic charge in solution. Dissolved organic matter was not included in the calculations due to uncertainties over its chemical composition and pH dependent charge, although we acknowledge that this would also lower free metal concentrations.

5.2.5. Metal sorption to soil

Sorption of Cu, Ni, Pb and Zn to the soil's solid phase was measured at sowing by batch extraction according to Namgay *et al*. (2010). Briefly, 25 ml of 0.01 M $Ca(NO_3)_2$ containing an equimolar (0.25 mM) concentration of $Cu(NO_3)_2$, $Ni(NO_3)_2$, $Pb(NO_3)_2$ and $Zn(NO_3)_2$ was added to 1 g of air-dried soil (sieved to <2 mm), the extracts shaken (75 rpm, 24 h) and the pH of the suspension measured. The samples were then centrifuged, filtered and metal concentration determined by ICP-OES as described above. Solid-to-solution metal partition coefficients (K_d) were

determined by dividing the amount sorbed to the solid phase (mmol kg^{-1}) by the equilibrium solution concentration (mmol l^{-1}).

5.2.6. Plant analysis

At plant harvest (45 d after sowing), shoot height and root length were measured, and dry weight determined after oven drying (80 °C, 48 h). Total As and metal (Cd, Cu, Ni, Pb, Zn) concentrations in leaves and roots were measured after microwave digestion with a Synthos 3000 (Anton-Paar, Graz, Austria) according to US-EPA (1995b). Prior to ICP-OES analysis, all samples were filtered as described above.

5.2.7. Statistical analysis

After checking for normality and homogeneity of variances, differences in treatments were compared by one-way ANOVA and Tukey HSD test using SPSS v.14 (SPSS Inc., Chicago, IL). T-tests were used to test for differences between biochar and ash chemical properties (Table 1).

5.3. Results

5.3.1. Chemical properties of biochar and wood ash

Properties of the biochar and ash are presented in Table 1. Overall, complete combustion caused metals to become concentrated in the ash relative to the partially combusted biochar with all measured parameters being significant ($P < 0.05$). Of particular note was the significantly higher EC and pH of the wood ash relative to the biochar and soils (Table 1 and 2).

Table 1. Chemical properties and total cation and heavy metal concentrations of the biochar and wood ash used in the experiments. Values represent average ± standard error (*n* = 3).

Parameter	Biochar	Wood ash
pH	9.77 ± 0.13	10.75 ± 0.02*
EC (mS cm^{-1})	1.66 ± 0.03	13.58 ± 0.15*
Na (g kg^{-1})	2.1 ± 0.3	14.5 ± 0.1*
K (g kg^{-1})	5.7 ± 0.6	24.5 ± 0.2*
Ca (g kg^{-1})	7.1 ± 1.5	31.0 ± 0.3*
Cu (g kg^{-1})	22.1 ± 2.4	198.6 ± 3.2*
Zn (g kg^{-1})	0.19 ± 0.02	2.97 ± 0.09*
As (mg kg^{-1})	9.01 ± 1.34	125 ± 2.62*
Cd (mg kg^{-1})	0.35 ± 0.12	5.25 ± 0.13*
Ni (mg kg^{-1})	1.7 ± 0.2	21.5 ± 1.1*
Pb (mg kg^{-1})	13.4 ± 1.3	76.5 ± 2.4*

* indicates statistically significant differences between the two amendments (Tukey HSD test, $P \leq 0.05$).

Cu, the dominant heavy metal, represented almost 2 and 20% of the total dry weight of the biochar and wood ash, respectively. In addition, the concentration of K, Na and heavy metals in the wood ash and biochar were significantly ($P \leq 0.05$) higher than in both soils (Tables 1 and 2).

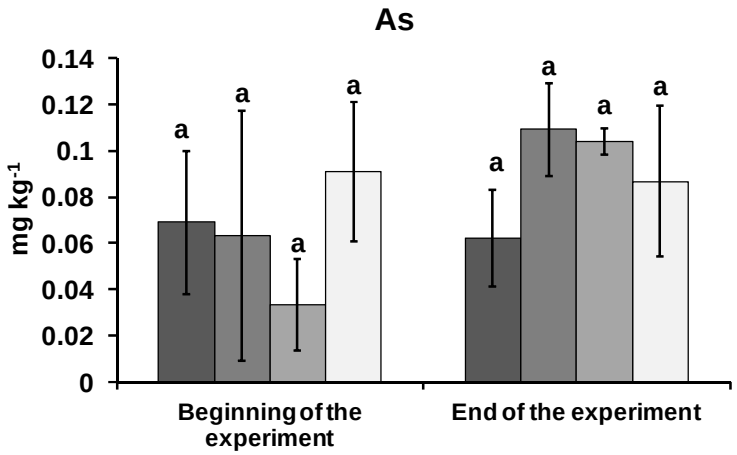
5.3.2. Soil properties and metal concentrations

Table 2. Properties of the two soils with or without addition of either biochar or wood ash used in the experiments. Values represent means ± standard errors (*n* = 5). Different letters indicate statistically significant differences among treatments within same parameter (Tukey HSD test, $P \leq 0.05$).

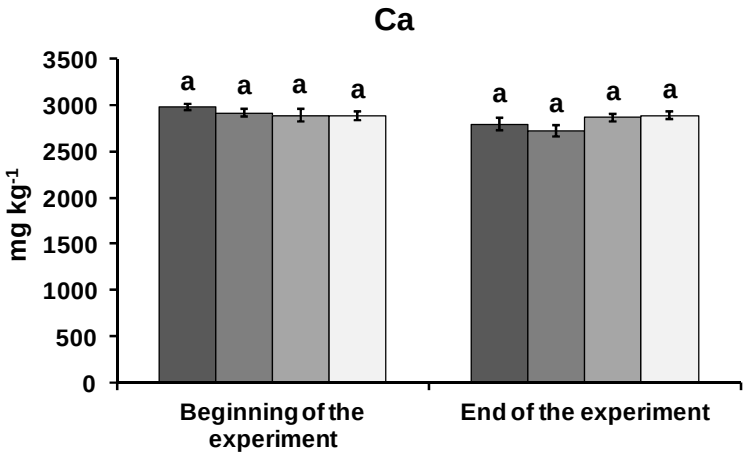
	Eutric Cambisol	Eutric Cambisol + biochar	Haplic Podzol	Haplic Podzol + wood ash
pH (initial)	6.02 ± 0.02$^{(c)}$	6.97 ± 0.01$^{(a)}$	4.95 ± 0.01$^{(d)}$	6.48 ± 0.04 $^{(b)}$
pH (end)	5.94 ± 0.07 $^{(b)}$	6.29 ± 0.09$^{(a)}$	4.73 ± 0.05$^{(d)}$	5.06 ± 0.08 $^{(c)}$
EC (μS cm^{-1})	20.4 ± 1.20 $^{(c)}$	31.3 ± 0.5 $^{(b)}$	16.7 ± 1.2 $^{(c)}$	39.5 ± 1.50 $^{(a)}$
Moisture (%)	17.2 ± 0.60 $^{(c)}$	15.2 ± 0.2 $^{(c)}$	27.5 ± 1.1 $^{(b)}$	20.4 ± 0.40 $^{(a)}$
Na (mg kg^{-1})	46.8 ± 2.51 $^{(c)}$	85.4 ± 2.45$^{(b)}$	46.7 ± 0.50$^{(c)}$	123 ± 1.68 $^{(a)}$
K (mg kg^{-1})	31.5 ± 0.17 $^{(d)}$	136 ± 3.67$^{(b)}$	16.0 ± 0.66 $^{(c)}$	158 ± 5.25 $^{(a)}$
Ca (mg kg^{-1})	5967 ± 33.0 $^{(b)}$	6817 ± 130 $^{(a)}$	28.3 ± 1.67$^{(d)}$	180 ± 2.89 $^{(c)}$
As (mg kg^{-1})	9.44 ± 0.12 $^{(b)}$	10.3 ± 0.72$^{(ab)}$	15.1 ± 1.15$^{(a)}$	14.5 ± 1.93 $^{(a)}$
Cd (mg kg^{-1})	0.65 ± 0.06	0.67 ± 0.06	0.71 ± 0.06	0.70 ± 0.11
Cu (mg kg^{-1})	11.5 ± 0.78 $^{(c)}$	181 ± 17.2$^{(b)}$	7.31 ± 0.57$^{(c)}$	351 ± 11.9 $^{(a)}$
Ni (mg kg^{-1})	9.18 ± 0.37 $^{(a)}$	8.21 ± 0.79$^{(ab)}$	7.51 ± 0.61$^{(ab)}$	6.66 ± 0.93$^{(b)}$
Pb (mg kg^{-1})	16.7 ± 0.79 $^{(a)}$	17.3 ± 0.95$^{(a)}$	9.09 ± 0.66$^{(b)}$	9.90 ± 0.56$^{(b)}$
Zn (mg kg^{-1})	37.6 ± 1.86	38.2 ± 3.76	33.2 ± 3.43	31.6 ± 4.12

The addition of both biochar and wood ash to soil resulted in an immediate increase in soil pH and EC (P<0.05), however, this response was not sustained and by the end of the experiment the pH had fallen back close to the unamended soil value (Table 2). The concentrations of Na, K and Ca were all increased in soils amended with both biochar and wood ash. There was also a significant increase in the concentration of total Cu following amendment by both biochar and wood ash (P<0.05). Although differences did exist between the two soil types, total concentrations of the potentially toxic elements Zn, Cd, As and Pb remained unaffected by the addition of either biochar or wood ash. In contrast, the concentration of total Ni was slightly reduced in both soil types following amendment by either biochar or wood ash.

5.3.3. Bioavailability of heavy metals and cations



A



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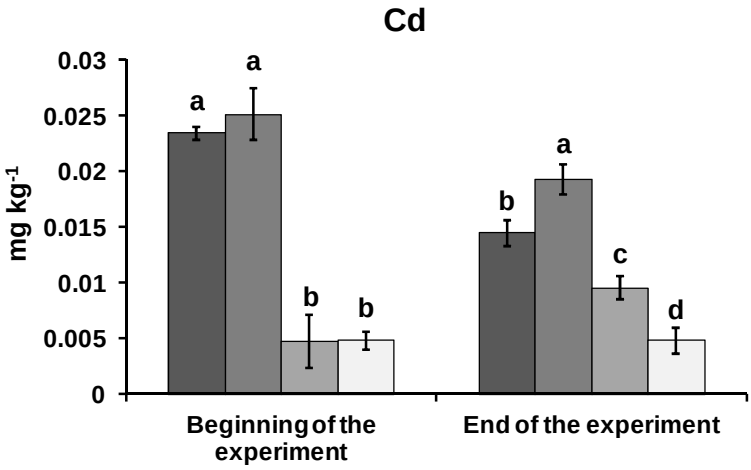

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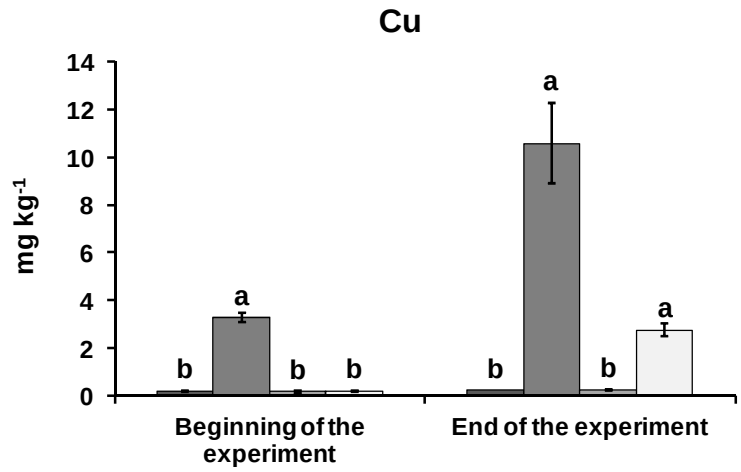

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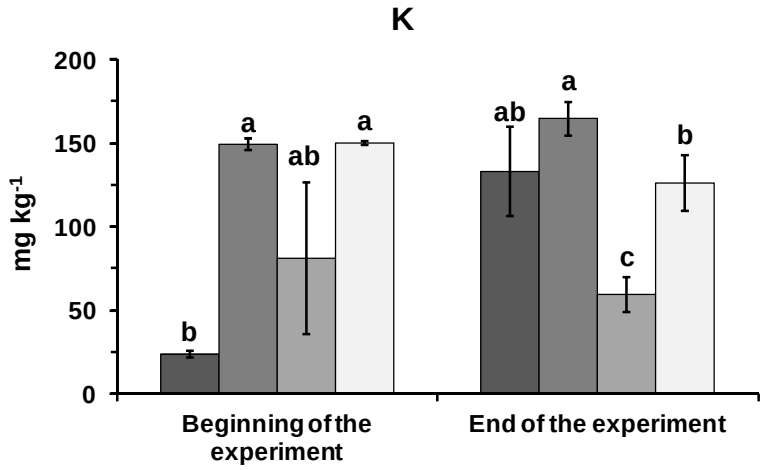

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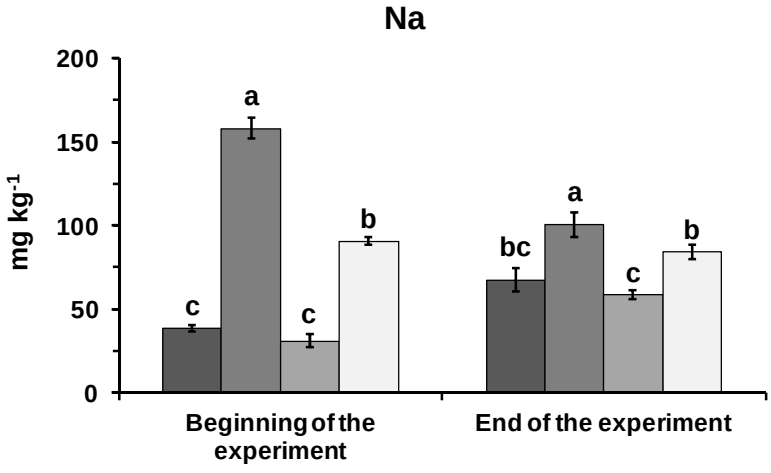

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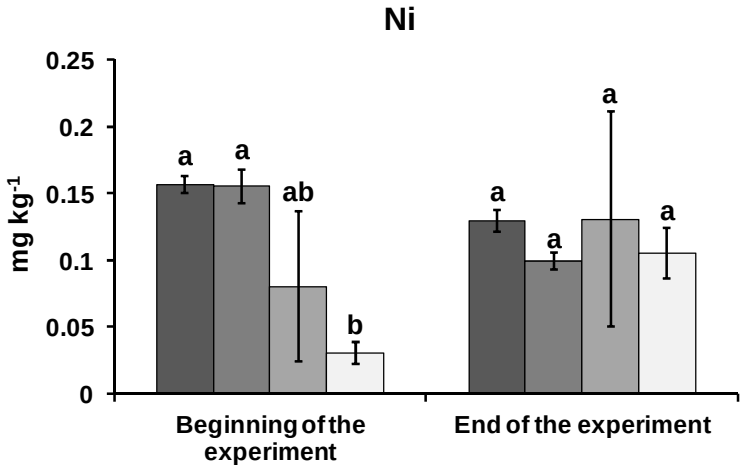

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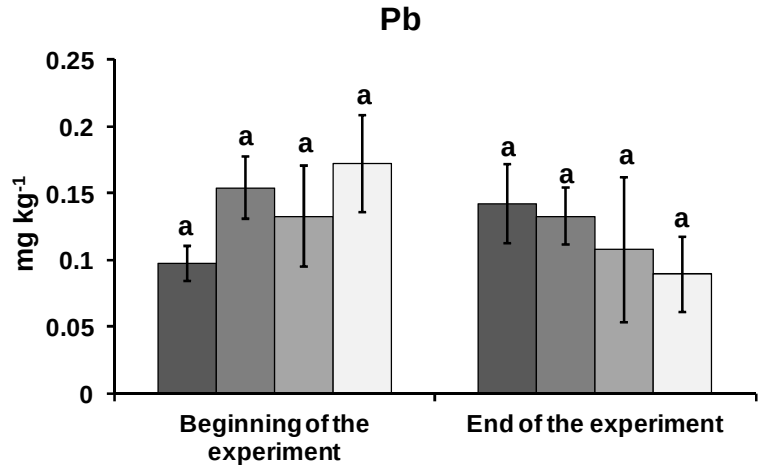

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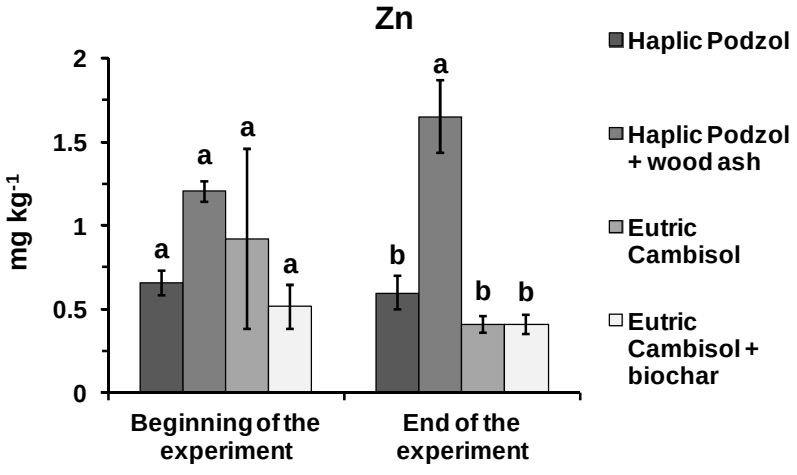

I

Figure 1. Nutrient and heavy metal bioavailability in two soils (Eutric Cambisol, Haplic Podzol) amended with either biochar or wood ash at the start and end of the experiment. Values represent means ± standard errors on a dry weight basis, whilst the different letters indicate statistically significant differences between treatments (Tukey HSD test, $P < 0.05$) for each date. The legend is the same for all panels.

Bioavailability of heavy metals and cations in soil measured at the beginning and end of the experiment are shown in Fig. 1. As expected, the concentration of bioavailable metals in soil were one to two orders of magnitude lower than the total metal concentrations reported in Table 1; however, most treatment trends appeared consistent between the two extractions.

Overall, the bioavailability of Ca, As, Pb, Zn, Cd and Ni remained relatively unchanged after the immediate addition of either biochar or wood ash to soil. After 45 d, however, the concentration of available Zn and Cd in soil had significantly increased in the wood ash treatment relative to the unamended control soil, whilst no such effect was observed in the biochar treatment. In contrast to the other heavy metals, there was an immediate large increase in bioavailable Cu after Cu-treated wood ash application to the Haplic Podzol soil. After 45 d, the availability of Cu had continued to increase in the wood ash-treated Haplic Podzol soil, whilst a significant increase was also apparent in the biochar-treated Eutric Cambisol ($P<0.05$).

There was also an immediate and significant increase in Na and K bioavailability in both soil types ($P<0.05$) following addition of biochar and wood ash, however, this effect was much less pronounced by day 45.

5.3.4. Metal speciation modelling

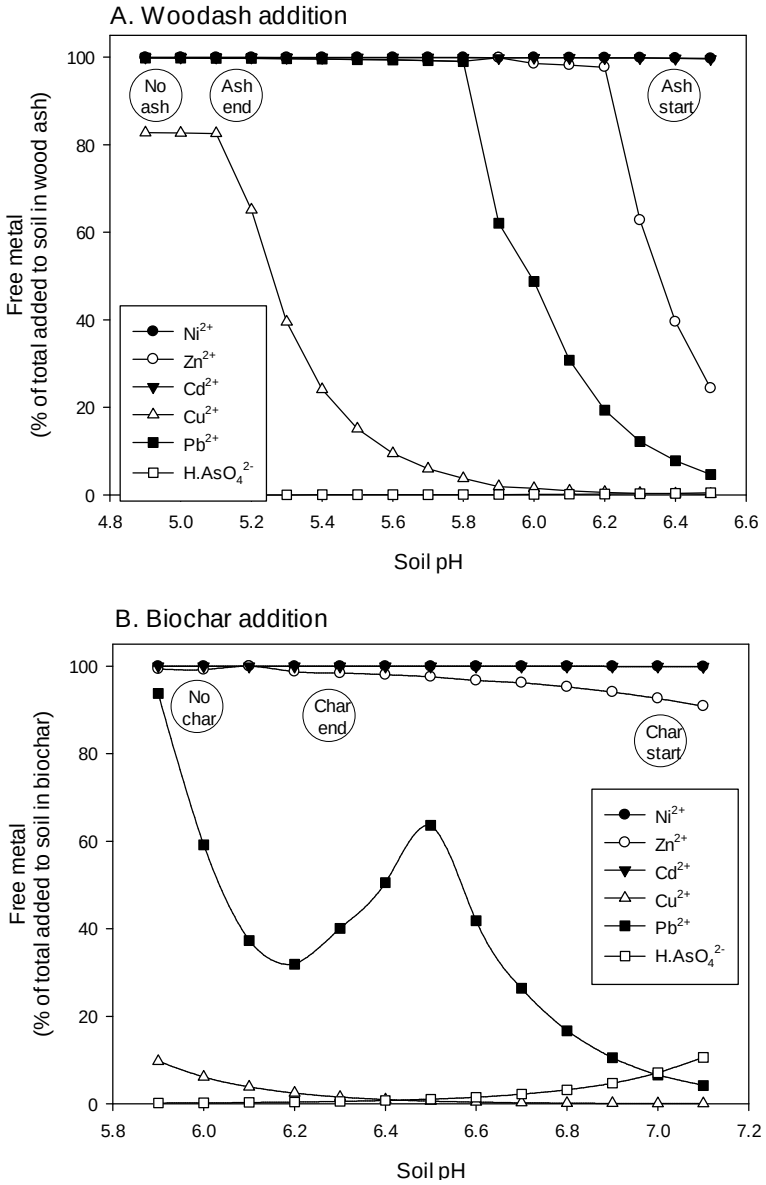

Figure 2. Predicted effect of the shift in soil pH on the proportion of free metals in solution after the application of either metal contaminated wood ash (Panel A) or biochar (Panel B) to soil. Metals not present in a free state were either present as metal-ligand complexes or had formed insoluble precipitates (e.g. $Cu(OH)_2$, $Zn_3(PO_4)_2$, $Pb_3(PO_4)_2$, $Cu_3(AsO_4)_2$). "No ash" indicates the pH of the unamended soil, "Ash start" indicates the soil pH immediately after wood ash addition and "Ash end" indicates the soil pH after 45 d (the labels follow a similar pattern for Panel B). The initial amount of metal in the soil follows that shown in Table 2 and is different for the two soils (Panel A, Haplic Podzol + wood ash; Panel B, Eutric Cambisol + biochar).

The predicted amount of free metal in soil solution in response to biochar or wood ash addition is shown in Fig. 2. In the Haplic Podzol the addition of wood ash and the resultant increase in pH was predicted to initially cause a large reduction in free Cu^{2+}, Pb^{2+} and Zn^{2+}-, whilst having no major effect on the availability of Ni^{2+}, $HAsO_4^{2-}$ and Cd^{2+}. Modeling indicated that most of the reduction in free metal concentration was due to the formation of insoluble metal complexes (e.g. $Cu(OH)_2$, $Zn_3(PO_4)_2$, $Pb_3(PO_4)_2$, $Cu_3(AsO_4)_2$), although some soluble metal complexes were also present. At the end of the experiment, when the pH effect of the ash had been reduced, it was predicted little effect of the ash on free metal availability in comparison to the unamended soil.

The liming effect of biochar in the Eutric Cambisol was also initially predicted to greatly reduce free metal concentrations (e.g. Cu^{2+}, Pb^{2+} and Zn^{2+}), however, it had no effect on Ni^{2+} or Cd^{2+}. Where reductions were predicted to occur, the effect became significantly diminished from the start to the end of the experiment. Due to the higher pH of the Eutric Cambisol in comparison to the Haplic Podzol, the addition of biochar was predicted to increase the solubility of As. Significant differences in metal response to pH between the two treatments was caused by differences in initial soil chemistry (e.g. base cation concentration) as well as the chemical nature of the amendment (e.g. S content).

5.3.5. Heavy metal sorption

Heavy metals were strongly adsorbed by both soils and followed the series Pb > Cu > Zn = Ni (Table 3). Generally, the presence of biochar and wood ash had little effect on the solid-to-solution partitioning of the metals as described by the $\log_{10} K_d$ values, with the exception of Pb sorption, which was increased in the presence of biochar and wood ash (P<0.01) and Zn in the presence of biochar (P<0.01).

Table 3. Solid-to-solution partition coefficients ($\log_{10} K_d$) describing the sorption of four heavy metals added to soil in the presence and absence of either Cu-treated wood derived biochar or ash. Values represent means ± SEM (n = 3). Letters indicate significant differences between treatments (P < 0.01).

	Solid-to-solution partition coefficient (log K_d) ($kg\ l^{-1}$)							
	Cu		Ni		Pb		Zn	
Eutric Cambisol	3.74	±0.01[a]	2.47	±0.01[b]	4.57	±0.05[b]	2.50	±0.01[b]
Eutric Cambisol + biochar	3.67	±0.05[a]	2.60	±0.02[a]	4.80	±0.07[a]	2.85	±0.03[a]
Haplic Podzol	2.53	±0.09[b]	1.91	±0.11[c]	2.91	±0.08[d]	1.72	±0.10[d]
Haplic Podzol + wood ash	2.74	±0.01[b]	2.00	±0.04[c]	3.63	±0.06[c]	1.85	±0.04[c]

5.3.6. Plant biomass and heavy metal uptake

Overall, plant growth was vigorous in the Eutric Cambisol while above- and below-ground growth remained very poor in the Haplic Podzol, irrespective of wood ash application, resulting in extremely stunted plants. Consequently, only biomass and metal contents were determined in the Eutric Cambisol grown plants. While the plants were of similar heights, the addition of biochar to the Eutric Cambisol reduced their total biomass by approximately 40% with a significant reduction observed in both root and shoot dry weights (P<0.05; Table 4). Cu-treated wood-derived biochar application had no significant effect on the accumulation of As, Cd, Zn, Ni and Pb in roots or leaves, but it did result in a 4-fold increase in foliar Cu concentrations and a 40-fold increase in total root Cu concentration (P<0.05). In addition, Cu accumulation (mg per plant) was higher in the biochar treated plants despite their lower biomass (data not shown).

Table 4. Growth characteristics and heavy metal concentration of sunflower shoots and roots after growth in Eutric Cambisol soil either amended with or without metal-contaminated biochar. Values are expressed on a dry weight basis and represent mean ± standard errors.

	Shoot		Root	
Parameter	Control	+ Biochar	Control	+ Biochar
Length (cm)	47.8 ± 2.1	48.0 ± 2.6	7.2 ± 0.6	7.1 ± 0.4
Biomass (g plant^{-1})	5.15 ± 0.67*	3.05 ± 0.39	0.73 ± 0.11	0.44 ± 0.07*
As (mg kg^{-1})	0.79 ± 0.12	0.68 ± 0.25	2.18 ± 0.53	3.37 ± 0.76
Cd (mg kg^{-1})	0.23 ± 0.13	0.12 ± 0.01	0.72 ± 0.29	0.83 ± 0.21
Cu (mg kg^{-1})	3.62 ± 0.33*	13.75 ± 2.16	5.11 ± 1.49	202 ± 52*
Ni (mg kg^{-1})	1.10 ± 0.09	0.89 ± 0.1	2.63 ± 0.73	3.89 ± 0.97
Pb (mg kg^{-1})	0.61 ± 0.09	0.43 ± 0.14	5.36 ± 0.67	5.46 ± 0.87
Zn (mg kg^{-1})	26.78 ± 6.30	21.25 ± 3.49	20.76 ± 6.46	41.88 ± 9.65

* indicates statistically significant differences between treatments (Tukey HSD test, $P \leq 0.05$).

5.4. Discussion

5.4.1. Biochar and wood ash metal content and potential for soil contamination

Reclaimed waste wood is being increasingly used for energy generation, however, this waste stream is also known to contain significant amounts of organic (e.g. PAHs) and metal contaminants (e.g. Cu, Cr, As). Recent work has shown that during pyrolysis or combustion, few of these metals are transferred into the bio-oil or are volatilized, thereby contaminant enrichment within the solid end-products (ash or biochar) is inevitable (Matsuura *et al*., 2009; Kim *et al*., 2012).

The type of feedstock and production process used to produce biochar and wood ash are important variables that can influence the final metal concentration (Demeyer, 2001; Reijnders, 2005; Atkinson *et al*., 2010). In this study, the

concentration of cations and metals in both the biochar and the wood ash were, in general, the same as those reported in the literature (e.g. Huang *et al*., 1992; Someshwar, 1996), although the concentrations of Cu and Na were higher than those previously reported (Etiégni and Campbell, 1991; Nieminen *et al*., 2005; Gaskin *et al*., 2008).

In the case of wood treated with Cu-preservative, high levels of Cu were found to be transferred to biochar (>20 g Cu kg^{-1}) and ash (~200 g Cu kg^{-1}), which were far in excess of those typically found in agricultural soils (0.001-0.1 g Cu kg^{-1}; McLaughlin, 2002), and above the maximum permissible limits for other common organic wastes (e.g. biosolids, 0.50-5.0 g Cu kg^{-1}; composts, 0.2 g Cu kg^{-1}; US-EPA, 1993; BSI, 2011). Based on the typical field application rates used here, ash and biochar would both result in high annual Cu loading rates (1000 kg Cu ha^{-1}) which are significantly above regulatory annual limits for Cu loadings to agricultural land (75 kg Cu ha^{-1} y^{-1}), but remain below lifetime loading rates (1500 kg Cu ha^{-1}) (US-EPA, 1993). The concentration of As, Cu and Zn in the wood ash were also higher than the recommended values for its use as a soil amendment (Risse *et al*., 2009). The resulting total soil Cu concentrations measured here after application (0.18-0.35 g Cu kg^{-1}) were much greater than soil Cu guidance limits designated as being of "negligible risk of environmental contamination" (0.01-0.07 g Cu kg^{-1}) and within the trigger limits for "unacceptable risk" (0.1-1.0 g Cu kg^{-1}), as designated by various EU member states (Carlon, 2007). These data, together with the plant growth results found here, suggests that Cu-preservative treated wood is not suitable for generating products destined for land application, and that other avenues should be sought for residue disposal (e.g. in construction materials; Cheah and Ramli, 2012).

If biochar or ash derived from non-treated wood is destined for land application, these results also indicate that contamination levels of waste wood streams by metal-treated timber should be set very low (≤ 1%) to minimize environmental risk. It should also be highlighted that separation of preservative treated wood from the bulk waste wood stream is logistically very difficult and still represents a major challenge to industry (Townsend *et al*., 2005).

5.4.2. Biochar and wood ash effects on soil pH

The addition of liming agents to grassland soils and the resulting increase in pH towards neutrality typically results in soil improvement due to an increase in nitrification, a reduction in rhizotoxic Al^{3+} and a concomitant increase in plant productivity (Kemmitt *et al*., 2006). Here it was found that both wood ash and biochar increased soil pH, but that this effect was relatively short lived. This decline in pH was ascribed to the gradual neutralization of the small amount of metal carbonates and oxides within the amendments. This pattern of pH response mirrors that seen in biochar field trials where an identical rate of application was used with this Eutric Cambisol soil (Jones *et al*., 2012). To achieve the optimal pH for sunflower production in the Haplic Podzol (pH 5.0→6.8) and Eutric Cambisol (pH 6.0→6.8), the calculated dose of $CaCO_3$ required would be 10 and 5 t ha^{-1} respectively, while for $Ca(OH)_2$ it would be 14 and 7 t ha^{-1} (Agricultural Lime Association, London, UK). Given the metal cation content of this ash and char (Ca, Na, K etc) it was estimated that it was added the equivalent of approximately 1 t $CaCO_3$ ha^{-1}, which explains why the amount added was insufficient to bring about a lasting change in soil pH. Overall, these results suggest that the positive liming effect of biochar and ash derived from Cu-contaminated wood does not offset the negative impacts of its high Cu content on soil quality and plant growth.

5.4.3. Effect of biochar and wood ash on metal cation availability

Heavy metals such as Cu^{2+} are known to strongly sorb to the surface of both soil organic matter and biochar, lowering free metal solution concentrations and limiting plant uptake (Ross, 1994; Namgay *et al*., 2010). Indirectly, the high pH of biochar and wood ash can also increase the pH-dependent negative charge on soil surfaces, stimulating further sorption as well as promoting metal precipitation (e.g. $Cu(OH)_2$) which readily occurs for several metals above pH 6.5 (Fig. 2; Lindsay, 2001). Biochar also contains significant amounts of dissolved organic C and HCO_3^- which may complex the free metals and render them non-phytotoxic (Jones *et al*., 2011). For these reasons, soil amendment with biochar has been advocated as a mechanism to remediate metal contaminated sites (Park *et al*., 2011a, b). The effect

of biochar on metal bioavailability, however, remains unclear as both increases and decreases in solution concentrations have been reported in the literature (Hua *et al*., 2009; Beesley *et al*., 2010; Fellet *et al*., 2011). In this study, the very high intrinsic Cu content of the char and ash clearly overwhelmed the immobilization capacity of the soil and biochar, resulting in phytotoxic concentrations being reached (Harden, 2011).

As regards the mobility of non-essential metals, like Cd and Ni, it was concluded that most of their bioavailable fraction originated from the soil rather than from the added char or ash. Due to the high cation content of the amendments, particularly K^+, Ca^{2+} and Cu^{2+} (Table 1), it was expected that their addition would stimulate desorption of the native Cd and Ni, increasing their bioavailability. This could occur by direct exchange of cations on sorption surfaces (e.g. Ca^{2+} for Cd^{2+}), and indirectly through cation displacement of H^+ from exchange surfaces, which lowers solution pH and makes metals more soluble (Namgay *et al*., 2010). The opposite response, however, was observed here, with biochar stimulating Cd retention at the end of the experiment. These findings contrast with those of Vergara and Schalscha (1992) and Vibhawari and Pandey (2010), who both found that high amounts of Cu^{2+} inhibited Cd^{2+} sorption in soil. The sorption and desorption reactions of mixtures of heavy metals, however, is a complex process dependent on both soil properties and competition between metals for sorption sites (Cerqueira *et al*., 2011). These results could suggest that Cd and Cu may occupy different sorption site profiles as reported by Yobouet *et al*. (2010).

It is clear from this trial that with the exception of Cu, biochar derived from Cu-treated wood appears to have minimal lasting effect on available heavy metal concentrations in soil. This supports the metal solubility predictions that showed little long term effects of pH on metal bioavailability. In contrast, Cu treated wood-derived ash application tends to increase the availability of native metals.

5.4.4. Effect of biochar and wood ash on metal oxyanion availability

Whilst raising the pH of the soil represents a major remediation option for most heavy metals (by rendering them insoluble), one of the negative consequences

of this can be an increased availability of oxyanions (e.g. As; Fig. 2; Jones and Healey, 2010). In contrast to the chemical equilibria predictions, however, experimentally there was little evidence to support an increase in As bioavailability in response to the biochar or wood ash induced rise in soil pH. Whilst the amount of As in these soils and biochar were within national guideline values for As in soil and organic wastes destined for land (1-150 mg As kg^{-1}; mean 40 mg kg^{-1}; Martin *et al*., 2009; Reimer and Cullen, 2009; US-EPA, 1994; Teaf, 2010), higher loading rates may occur with wood treated with As-based preservatives (e.g. Cu-Cr-As or monosodium methanearsonate). Although the feedstock material used here possessed a relatively low As concentration (<0.05 g kg^{-1}), As-treated wood typically contains between 1 to 18 g As kg^{-1}, a concentration that is similar to that of Cu in the wood used here (Hingston *et al*., 2002). Upon pyrolysis or incineration, some As will volatilize, however, significant quantities will be retained in the ash and char (ca. 30-40% at 500-600 °C reducing to 10-20% at 850-1500 °C; Gray *et al*., 2001; Kim *et al*., 2012). At these higher concentrations (1-18 g kg^{-1}), it was estimated that the amount of As added to soil within ash or biochar will result in soil concentrations ranging from 1 to 100 mg As kg^{-1} exceeding regulatory limits for soils in many countries and effectively rendering the soil contaminated. In addition, the As sorption capacity (S_{max}) of most soils will be readily saturated (S_{max} typically 0.01-0.1 mg As kg^{-1}; Burns *et al*., 2006), leading to high solution concentrations and a risk of leaching to groundwater. It should be noted, however, that Cu readily precipitates with As, and this may offer some protection against leaching and plant uptake.

As wood-derived biochars are predominantly negatively charged, their capacity for sorbing arsenic is very low in comparison to metals such as Cu and Zn (Beesley and Marmiroli, 2011). The lack of potential for the added biochar to help lock up As is supported by a range of studies showing little effect in reducing soil As concentrations or plant As uptake (Beesley *et al*., 2011). Although Cu-Cr-As treatment is, or has been, phased out in many countries, it can be expected to be present in many waste wood streams for decades to come, and this also provides another reason for not recommending the use of preservative treated wood for biochar production.

5.5. Conclusions

These results show that waste wood materials containing high levels of heavy metals should not be used to produce biochar and wood ash intended for use as soil amendments. The liming effect on soil following such amendments together with their potentially high surface charge are insufficient to reduce metal bioavailability and plant uptake, particularly when the products themselves have high metal contents. The study has confirmed that even at normal addition rates, such as those used here, contaminated wood ash and biochar increase metal (e.g. Cu) bioavailability, and this is of concern for leaching and subsequent groundwater and food chain contamination. Their application to soil may also exacerbate the bioavailability of previously non-bioavailable oxyanions such as As. When biochar and ash are derived from wood treated with Cu-based preservatives, extremely high Cu concentrations in soil and reduced plant biomass due to Cu toxicity are easily observable. Waste wood, however, may also contain significant quantities of other metals (e.g. Cr, As, Pb), which will also lead to the exceedance of statutory limits for soil contamination. Feedstock quality is therefore of paramount importance for the effect of biochar and wood ash on soil and plant productivity and to protect human and environment health. Overall, it was concluded that whilst waste streams containing preservative treated wood are suitable for energy recovery, the ash or biochar residues produced from this process may not be suitable for land disposal.

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Conclusions

Phytostabilization of heavy metals is a green technology that can be used for immobilizing toxic elements either in the roots or in the rhizosphere. However, this study has evidenced that the efficiency of this technology is greatly affected by the metal itself and soil conditions, so that each polluted site must be carefully characterized before any treatment is applied.

This study showed that *in-planta* phytostabilization of heavy metals with annual plant species is feasible in their taproots and allows to effectively immobilize relatively high percentages of many elements (Co, Cr, Cu, Mn, Ni, Pb) in the long-term. Stabilization was effective in the relatively short-lived organic residues of rapeseed taproots, but longer-lived residues, like taproots of polyannual species or coarse roots of woody plants may ensure better results. These would allow to recover higher amounts of metals and stabilize them for longer time, due to the larger root size and biomass and their higher resistance to degradation. As a result, the restoration of polluted soils through woody plants might be achieved in shorter time span than through the roots of herbaceous species, although the latter may be more yielding in the first years. The time span of soil restoration is an important key issue for determining the feasibility of remediation, because it is related to the process costs, and green technologies generally require long time to be effective. Indeed, phytostabilization can be efficiently combined with phytoextraction, increasing the overall efficiency of the process. Compared with metal phytoextraction, *in-planta* phytostabilization does not produce polluted aboveground residues, and does not require any additional cost for the treatment or disposal of biomasses. In this view, phytostabilization might be a promising and cost-effective technique for limit metal accumulation in the food chain.

Ex-planta phytostabilization through organic amendments appeared more complex, since these materials increase the bioavailability of some metals, as evidenced in a pot-experiment with forage sorghum. The effects on metal mobility appeared unrelated to the source of organic matter, therefore it is concluded that both animal and plant residues can be suitable for the production of amendments intended for soil remediation. Higher metal retention can be achieved when the amendment is subjected to stabilization processes which increase the humification rate and the

contents of humic substances, although attention should be paid to the contamination level of the organic feedstock.

Essential components of organic amendments are humic compounds, which presence was associated to better plant growth belowground. Humic compounds and dissolved organic matter (DOM) can also respectively reduce and increase metal mobility through complexation processes. In particular, high levels of DOM increase soil Cu bioavailability, while relatively high amounts of humic compounds have an opposite effect, Although the mobility of other metals like Cd and Pb, which have only weak interactions with organic matter, is influenced by other factors, the type of organic amendment should be chosen carefully when used in phytoremediation.

If stabilization is intended to be achieved through the addition of organic amendments, the changes in the chemical composition of the amendments themselves is a crucial issue, since in the middle-long term the chemical composition of amendments changes as well as their ability to interact with soil and metals, as it was shown in a field-experiment with biochar. This aspect is not usually taken into account because much literature refers to short-term studies, suggesting the need of further investigation.

Overall, stabilization of metals in the long term can be achieved through different methods, but periodic controls and a deep knowledge of the polluted site are needed to ensure the effectiveness of the restoration.