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Changes in the heavy metal solubility of two contaminated soils after heavy metals phytoextraction with *Noccaea caerulescens*

Isabel Martínez-Alcalá, M. Pilar Bernal, Carlos de la Fuente, Dora Gondar, Rafael Clemente*

Department of Soil and Water Conservation and Organic Waste Management, CEBAS-CSIC, Campus Universitario de Espinardo, Murcia, Spain

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ABSTRACT

Hyperacumulator plant species, such as *Noccaea caerulescens*, have been deeply studied due to their use in phytoextraction techniques, although the fate of the metals remaining in the roots at the end of the remediation process is still uncertain. Here, germination and growth, metal accumulation in plant tissues and degradation of roots remaining in the soil after harvest have been studied in two contaminated soils from an area affected by a toxic pyritic sludge spillage. Specially designed pots allowed the separation of the bulk soil and rhizosphere, where heavy metals fractionation in soil was determined at the end of the growing period. High Cd and, especially, Zn concentrations in the aerial parts of the plants were found, although the bio-concentration factors (BCF) were higher for Cd (13–34) than for Zn (2.37–4.34). For both soils, the soluble and exchangeable ($CaCl_2$-extractable) concentrations of Fe and Mn were higher in the rhizosphere than in the bulk soil, while Zn and Cd concentrations were greater in the bulk soil. After plant harvesting, the degradation of heavy metal enriched roots in the soil was studied as well as the effect of this process on soil metal solubility. The soluble concentrations of Cu and Mn were higher in the soils with roots that in the corresponding soils without roots. Nevertheless, the amount of heavy metals released to the soil after root degradation (8–14% of their organic C was decomposed) was rather low (below 0.1 $\mu g\,g^{-1}$), showing the feasibility of the use of *N. caerulescens* for phytoextraction.

1. Introduction

Around 400 plant species that are adapted to extreme soil metal environments and accumulate high levels of heavy metals in their shoots exist, mainly distributed in families such as the Asteraceae, Brassicaceae, Caryophyllaceae, Poaceae, Violaceae and Fabaceae (Milner and Kochian, 2008). This phenomenon is known as hyperaccumulation, a term first coined by Brooks (1977) for plants that grow in metalliferous soils that are able to tolerate metals and accumulate them in their aerial parts to high concentrations and show no symptoms of toxicity. Brassicaceae is the most relevant of all these families, with 87 species classified as metal hyperaccumulators. For example, *Noccaea caerulescens* has some ecotypes able to accumulate as much as 30,000 $\mu g\,g^{-1}$ Zn and 10,000 $\mu g\,g^{-1}$ Cd in the shoots without any sign of toxicity (Milner and Kochian, 2008).

Heavy metal hyperaccumulation in *N. caerulescens* appears to be mediated by four physiological mechanisms (Milner and Kochian, 2008): stimulated metal influx across the root cell plasma membrane; reduced metal sequestration in the root vacuole; increased loading into the xylem for transport to the shoots; stimulated metal influx across the leaf cell plasma membrane and sequestration in the leaf vacuole. As a consequence, metals remaining in the roots after phytoextraction procedures have been scarcely taken into account regarding their possible reincorporation into the soil or soil solution.

Some hyperaccumulator species provoke changes in the rhizosphere to increase metal availability; these may involve the exudation of organic acids by the roots (Kidd et al., 2009; Puschenreiter et al., 2005). However, in hydroponic studies, where all the Cd and Zn was readily available, *N. caerulescens* was also able to accumulate much more Cd and Zn than *Thlaspi arvense* (a non-accumulator species)—even at low metal concentrations (Pence et al., 2000). In addition, Zhao et al. (2001) found no significant differences in organic acids exudation from the roots between *N. caerulescens* and *T. arvense*, so it seems that *N. caerulescens* does not have any special ability, compared to non-accumulator species, with regard to increasing metal availability in the rhizosphere. In agreement with this, Hammer et al. (2006) found that the fact that *N. caerulescens* accumulated greater amounts of metals than a non-hyperaccumulator species was not associated with an increase

* Corresponding author. Tel.: +34 968396385; fax: +34 968396213.
E-mail address: rclemente@cebas.csic.es (R. Clemente).

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in the pool of metals in the soil solution. Whiting et al. (2001) found that axenically grown plants of *N. caerulescens* accumulated 25–42% less Zn in the shoots than those grown in non-sterile soil, demonstrating that rhizosphere microbes play a key role in metal availability for this species. Also, Aboudrar et al. (2007) found a structural effect of the rhizosphere of *N. caerulescens* on bacterial communities that resulted in a greater microbial tolerance to Ni. All these findings manifest the interest in studying the changes in the rhizosphere of hyperaccumulator species in order to elucidate the processes involved in metal uptake and accumulation by the plants.

As a consequence, numerous phytoremediation studies can be found in the literature regarding hyperaccumulator species; most of them focused on trace elements uptake by the plants and the immediate effects in the soil. But, information about what happens in the rhizosphere of hyperaccumulator species like *N. caerulescens* and about what happens in the soil after root degradation is scarce. Therefore, the aim of this work was to study two different issues related to the use of *N. caerulescens* in phytoextraction: (i) the effects of the plants on the distribution of heavy metals in the soil directly affected by the roots (rhizosphere); and (ii) the potential release of soluble forms of heavy metals from the roots into the soil solution as the roots degrade in the soils after the harvest of the aerial parts of the plants.

2. Materials and methods

2.1. Soil characteristics

Two soils were selected from the same site (37°26′15″N, 06°13′05″W); these were affected differently by the pyritic mine sludge spillage that took place at the Aznalcóllar mine (Seville, Spain) in 1998. Both were non-calcareous loamy soils (19.7% clay, 34.3% silt and 46% sand) classified as Typic Xerofluvent (American Soil Taxonomy), and had similar physico-chemical properties but different degrees of heavy metal contamination (Table 1). The first (soil A) showed lower concentrations than the second (soil B), the total Cd, Cu, Pb and Zn concentrations of which were above the EU limits for agricultural soils when sewage sludge is applied (CEC, 1986), and can be considered slightly and moderately contaminated, respectively (Kabata-Pendias, 2001). Composite soil samples were collected from the top 20 cm, air-dried and sieved (<2 mm) before their use in the experiment and the corresponding analyses.

2.2. Experimental design

2.2.1. Rhizopots experiment

Specially designed rhizopots, whose detailed description has been previously reported (Martínez-Alcalá et al., 2009), were used for the separation of the rhizosphere from the bulk soil. Briefly, the rhizopots consisted of two compartments, an upper compartment (a polyvinyl chloride cylinder), which held the soil (170 g) where plants grew, and a lower part (a small plastic pot) that was considered the bulk soil (120 g). The rhizosphere was considered as the soil layer (2-mm-thick, 5 g) situated between the two compartments, which were separated by a nylon monofilament gauze (200-μm pore size; Sefar Inc., Switzerland) introduced in the rhizopots after the complete development of a root system in the upper part.

Seeds of *N. caerulescens* (J. & C. Presl) F.K. Mey (formerly *Thlaspi caerulescens* J. & C. Presl) were collected at a metalliferous site (High Tor, Derbyshire, UK); this population is known to be able to accumulate more than 1% Zn in its shoots (Walker and Bernal, 2004). No other accessions were considered for the present experiment due to limited accessibility and to focus on the effect of the degradation of plant roots with elevated metal concentrations. The seeds were surface-sterilised (10% HClO for 30 min), washed thoroughly with distilled water and then germinated in a plastic container, on sand moistened with 0.5 mM $CaSO_4$, in an incubator at 28 °C for 16 days. Then, five plants per rhizopot were planted and maintained in a growth chamber (light/dark regime 16/8 h, temperature 25/17 °C day/night, and relative humidity 70/70%) all over the experiment. The plants were watered with deionised water from the base of the rhizopots (to minimise metal and nutrient leaching) to approximately 70% of the soil water-holding capacity. No fertilisers were applied to the soils.

A total of 60 rhizopots distributed in three trays were run per soil and placed in the growth chamber, following a randomised block design. After 116 days of growth in the rhizopots (once the roots were fully developed and had reached the bottom of the upper cylinder), rhizosphere soils were added between the upper and the lower parts of the rhizopots; 16 days later (132 days of growth), the rhizosphere soil was sampled—combining the intermediate layers of the rhizopots from each tray in composite replicate samples ($n = 3$). At that moment, whole plants (100 per replicate tray) were harvested and their roots and shoots separated ($n = 3$). The shoots were rinsed with distilled water whereas the roots were first thoroughly washed with tap water, then with distilled water under sonication (7.5 min) to remove soil particles and finally with 0.1 mM $SrCl_2$ (30 s) to remove metals adsorbed on the root surface. The fresh and dry (70 °C) weights of the roots and shoots were determined before being ground for analysis. Only roots coming out from the bottom of the upper part of the rhizopot (in contact with the rhizosphere soil and easy to clean as very few soil particles adhered to them) were used for chemical analysis. The upper part (soil plus roots) of four rhizopots – out of 20 – from each tray was used for the incubation experiment, and the remaining 16 (per tray) were combined and used for chemical analysis of the soil after separation of the roots.

2.2.2. Incubation experiment

The intact upper part of the rhizopots (where plants had grown; $n = 12$) and 120 g of soil without roots taken from the lower compartment (bulk soil; $n = 12$) were placed separately in 0.5 l incubation vessels. The root density, calculated in the 16 rhizopots from each tray not used in the incubation, was similar in the two soils after plant growth (1.82 and 1.76 g kg^{-1} in soil A and soil B, respectively). The moisture content of the soils was taken to 60% of their water-holding capacity, and the incubations were carried out in the dark, in a temperature-controlled incubator at 26 °C, for 56 days. The CO_2 evolved was trapped in a beaker containing a NaOH (Clemente and Bernal, 2006), and determined every two days during the first week, and then weekly until the end of the experiment (56 days), by titration of the NaOH solution with HCl in an excess of $BaCl_2$, and expressed as μg of CO_2–C per gram of soil. The amount of CO_2–C coming from roots degradation was calculated by comparing CO_2–C evolved in the soil with roots with that in the

Table 1
Physico-chemical characteristics of the soils. Values in brackets correspond to $CaCl_2$-extractable concentrations (μg g^{-1}).

	Soil A	Soil B
pH	6.80	7.09
EC (dS m^{-1})	0.78	2.30
OM (%)	1.8	2.2
TOC (g kg^{-1})	10.4	12.9
TN (g kg^{-1})	1.14	1.00
$CaCO_3$ (%)	<0.5	<0.5
Available P (μg g^{-1})	21.9	11.2
Cd (μg g^{-1})	0.9 (0.3)	2.3 (0.3)
Cu (μg g^{-1})	103 (0.1)	200 (0.3)
Fe (mg g^{-1})	42.1 (0.5)	45.2 (0.4)
Mn (μg g^{-1})	834 (0.1)	1110 (9.1)
Pb (μg g^{-1})	141 (0.5)	500 (<0.01)
Zn (μg g^{-1})	360 (0.7)	890 (2.8)

bulk soil (without roots), and expressed as a percentage of the total organic carbon (TOC) of the roots. After 56 days of incubation, the soils were air-dried, ground and sieved prior to analysis.

2.3. Analytical methods

General soil analyses were carried out as described by Martínez-Alcalá et al. (2009). The pseudo-total concentrations of heavy metals (Cd, Cu, Fe, Mn, Pb, Zn) in soils (metals bound strongly to silicate minerals were not dissolved) and total concentrations in plants were determined by flame atomic absorption spectrometry (AAS) in a UNICAM 969 atomic absorption spectrometer (Thermo Elemental, Cambridge, UK), after nitric–perchloric acid (2:1) digestion for at least 2 h to a maximum of 210 °C. The $CaCl_2$-extractable metals (0.1 M $CaCl_2$ 1:10 w/v, 16 h) and plant macronutrient concentrations were determined by ICP-OES (Iris Intrepid II XDL, Thermo Scientific). A sequential extraction of soil metals (McGrath and Cegarra, 1992) was carried out in the soils (bulk and rhizosphere) at the end of the plant growth experiment. This had the following steps: (i) 0.1 M $CaCl_2$ (1:10 w/v, 16 h), metals in soil solution and in exchangeable forms; (ii) 0.5 M NaOH (1:10 w/v, 16 h) followed by *aqua regia* digestion of the extracts, metals associated with soil organic matter; (iii) 0.05 M Na_2H_2EDTA (1:10 w/v, 1 h), metals mainly in the carbonate fraction; and (iv) acid digestion of the remaining soil with *aqua regia*, residual metals. The soil microbial biomass-C (B_C) (fumigation-extraction procedure, Vance et al., 1987) and water-soluble (1:10 w/v) organic carbon (C_W), were determined in a TOC-V Analyzer (Shimadzu, Tokyo, Japan). Soil pH and redox potential (*Eh*) were determined in saturated soil pastes. The biomass-N (B_{NIN}) was also determined in the soils as the difference between the ninhydrin-reactive N in fumigated and non-fumigated soil extracts (Joergensen and Brookes, 1990). Soil available-P concentrations were determined colorimetrically (0.5 M $NaHCO_3$, 1:10, w/v). All the concentrations were adjusted to values for oven-dried soil (12 h at 105 °C). Total P concentrations in plant material (oven-dried tissues) were determined colorimetrically after nitric–perchloric acid digestion (210 °C), and those of soluble inorganic anions (Cl^-, NO_3^-, PO_4^{3-}, SO_4^{2-}) were determined in water extracts (1:200, w/v) by ion chromatography (Dionex DX500). All the chemical analyses were performed at least in duplicate.

The bioconcentration factor (BCF) and the translocation factor (TF) were calculated as the ratio between the metal concentrations in the shoots and soils (pseudototal), and between the shoots and roots, respectively.

Although these two parameters are usually related, the TF reflects the ability of the plant to transfer metals to above-ground organs, while the BCF shows the portion of the pollutant present in the soil that actually accumulates in the aerial parts of the plant (Baker, 1981). Both parameters strongly depend on the plant species and are used for their classification according to their metal accumulation properties, and to assess their potential use in phytoremediation (Baker, 1981).

2.4. Statistical analysis

The data were subjected to one- and/or two-way ANOVA using the software SPSS 17.0. Data regarding CO_2–C evolution from the soils were fitted to a first-order kinetic equation by a non-linear least-square procedure (Marquard–Levenberg algorithm), using the SigmaPlot computer programme. The model is expressed according to the following equation:

$$C_m = C_0 \times (1 - e^{-k \times t}),$$

where C_m is the mineralised-C per gram of dry soil ($\mu g\, g^{-1}$) at a time t (days), C_0 the potentially mineralisable-C ($\mu g\, g^{-1}$) and k the rate constant (day^{-1}). The residual mean square (RMS) and F-values were calculated for the statistical significance of the curve fitting.

3. Results

3.1. Plant growth and nutritional status

The yield of *N. caerulescens* (aerial parts) was higher ($P < 0.05$) in the slightly polluted soil (soil A, 0.65 ± 0.07 g DW pot^{-1}) than in the moderately polluted soil (soil B, 0.51 ± 0.05 g DW pot^{-1}), although the fresh to dry weight ratios were similar for the two soils—as was root growth (0.31 ± 0.05 g DW pot^{-1} in soil A and 0.30 ± 0.06 g DW pot^{-1} in soil B).

The concentrations of nutrients (total N, K) and Na (of interest in high-EC soils like the ones in the present experiment) in the aerial parts of the plants were also significantly higher in plants grown in soil A (51, 37 and 0.4 mg g^{-1} DW, respectively) than in those grown in soil B (23, 29 and 0.1 mg g^{-1} DW, respectively; Table A1, Supporting information). Similar results were found in roots, with higher total N, P, Ca and Na concentrations for soil A than for soil B, with the exception of K, whose concentration in soil B (26 mg g^{-1} DW) was greater than in soil A (18 mg g^{-1} DW). Plants from both soils showed high sulphate concentrations in their aerial parts (22,484 and 16,009 $\mu g\, g^{-1}$ DW in soils A and B, respectively; Table A2, Supporting information) while the concentrations of NO_3^- and PO_4^{3-} in shoots and roots were much higher in plants grown in soil A, compared to soil B.

3.2. Heavy metals concentrations in the plants

Noccaea caerulescens accumulated Cd and, especially, Zn to high concentrations in the shoots (Fig. 1), while the rest of the heavy metals determined accumulated mainly in the roots, in both soils. The Pb and Zn concentrations in both tissues (roots and aerial parts) were significantly higher in soil B than in soil A. The bioconcentration factor (BCF) in *N. caerulescens* was greater than 13 for Cd and greater than 2 for Zn, in both soils, and showed no significant differences (data not shown). The Zn translocation factor (TF) in plants

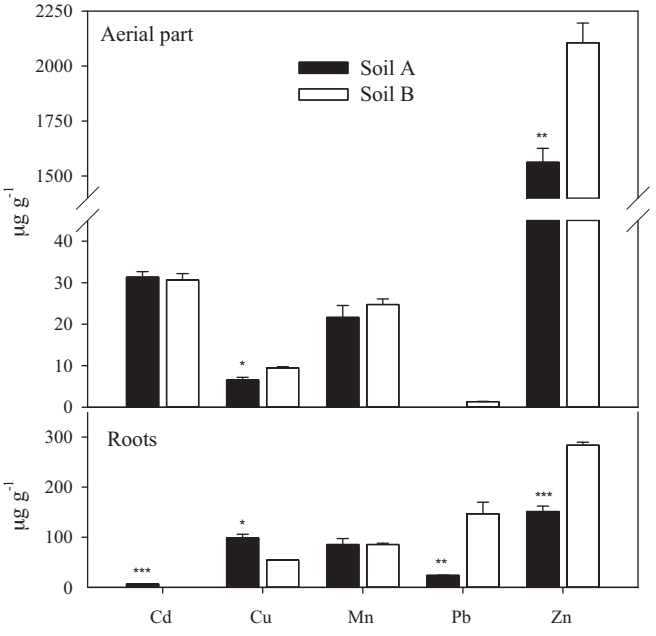

Fig. 1. Heavy metals concentrations in shoots and roots of *Noccaea caerulescens* ($\mu g\, g^{-1}$) grown in the less polluted (soil A) and the moderately polluted soil (soil B) ($n = 3$). *, **, ***: significant at $P < 0.05$, 0.01 and 0.001, respectively.

grown in the low metal soil (A) was 10.3, and in the moderately contaminated soil (B) it was 7.43, while for Cd the TF was lower in soil A (4.85) than in soil B (12.4). The Cu, Pb and Zn concentrations in the aerial parts of the plants were significantly higher in plants grown in soil B than in those raised in the low metal soil A (Fig. 1). In the roots, the concentrations of Pb and Zn were also higher in soil B than in soil A, while the opposite occurred for Cu and Cd.

3.3. Rhizosphere and bulk soil characteristics

In soil A, the redox potential (*Eh*) was significantly higher in the rhizosphere than in the bulk soil, while the water soluble organic-C (C_W) was higher in the bulk soil (Table 2). In soil B, the microbial biomass-C and -N and C_W values were significantly higher in the rhizosphere than in the bulk soil, and no significant differences regarding soil pH and *Eh* were found.

Regarding metal extractability in the soils, the overall soluble and exchangeable ($CaCl_2$-extractable) concentrations were very low (below 1 mg kg^{-1}), and only in soil B were the Fe and Mn concentrations significantly higher in the rhizosphere than in the bulk soil (Table 3). In soil A, the NaOH-extractable concentrations of Zn were significantly lower in the rhizosphere (3.70 $\mu g\,g^{-1}$) than in the bulk soil (4.79 $\mu g\,g^{-1}$) (Fig. 2). For both soils, the greatest effect with respect to Cu, Fe, Mn, Pb and Zn fractionation was found in the EDTA-extractable fraction, with lower concentrations in the rhizosphere than in the bulk soil, especially in the moderately contaminated soil (Fig. 2).

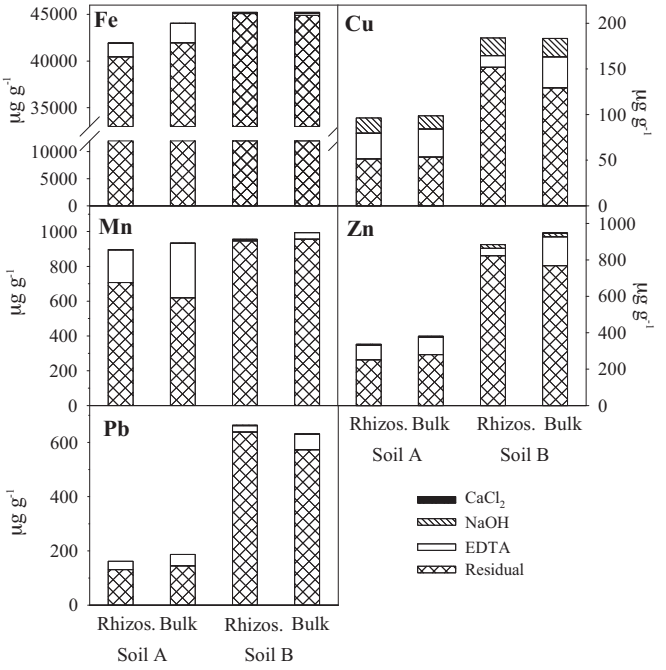

Fig. 2. Heavy metals fractionation (sequential extraction) of Cu, Fe, Mn, Pb and Zn (Cd concentrations were considered too low for fractionation) in the bulk soil and rhizosphere of *Noccaea caerulescens* ($n = 3$).

3.4. Root degradation and release of heavy metals in the soils

The CO_2–C production rate was slightly higher in the soils with roots than in the soils without roots, mainly in soil A, and it showed, in all cases, a fast decrease during the first 10 days—becoming much lower and almost constant by the end of the incubation (Fig. 3a and b). Nevertheless, the mineralised-C at the end of the experiment was higher in soil with roots (304 and 442 $\mu g\,g^{-1}$ for soils A and B, respectively) than in the corresponding soil without roots (267 and 402 $\mu g\,g^{-1}$ for soils A and B, respectively), indicating certain degradation of the root tissues (Fig. 3c and d). The cumulative CO_2–C evolution followed a first-order kinetic function, in which the mineralised-C was proportional to the potentially mineralisable carbon (C_0). The parameters of the kinetic model showed that the C_0 was higher in the moderately polluted soil than in the less polluted one (Fig. 3c and d), although the latter showed a higher rate constant (k). At the end of the incubation, the mineralised-C from roots was higher in soil B than in soil A (14.4 and 7.9%, respectively), despite the fact that the root density was similar in both soils. The greater B_C concentrations in the moderately polluted soil may have been responsible for the higher biodegradability of roots in that soil.

At the end of the incubation, the soluble and exchangeable ($CaCl_2$-extractable) concentrations of heavy metals in the soils were determined in order to study the influence of root degradation on heavy metals solubility (Table 3). In soil A, significantly higher concentrations of Cu and, especially, of Mn were observed in the soil with roots with respect to the bulk (no roots) soil, while the opposite was true for Pb and Zn. In soil B, statistically significant differences were only observed for the Pb concentrations, which were higher in the soil with roots than in the bulk soil without roots.

Taking into account the root heavy metals concentrations, the quantity of roots present in the soil and the carbon mineralised after 56 days of incubation (as a percentage of root degradation), the release of heavy metals from the roots was estimated. The net supply of Cu, Mn, Pb and Zn to the soils was very low (<0.1 $\mu g\,g^{-1}$) in both soils, while that of Fe was greater, it being significantly higher in soil B (1.89 $\mu g\,g^{-1}$) than in soil A (1.07 $\mu g\,g^{-1}$).

Table 2
Soil properties in the rhizosphere and bulk soil (mean ± se, $n = 3$) of the plant growth (rhizopots) experiment: pH, redox potential (*Eh*), water soluble organic-C (C_W), soil microbial biomass-C (B_C) and biomass ninhydrin reactive-N (B_{NIN}).

		pH	*Eh* (mV)	C_W ($\mu g\,C\,g^{-1}$)	B_C ($\mu g\,C\,g^{-1}$)	B_{NIN} ($\mu g\,N\,g^{-1}$)
Soil A	Rhizosphere	6.5 ± 0.13	304 ± 3.00	46.3 ± 1.45	53 ± 12.3	5.9 ± 0.52
	Bulk Soil	6.8 ± 0.03	286 ± 1.00	60.3 ± 4.70	62 ± 2.64	7.4 ± 0.45
	ANOVA[a]	N.S.	*	*	N.S.	N.S.
Soil B	Rhizosphere	7.6 ± 0.04	249 ± 2.89	12.9 ± 0.19	113 ± 4.00	23 ± 2.33
	Bulk Soil	7.4 ± 0.08	251 ± 5.24	8.0 ± 0.20	68 ± 7.80	13 ± 1.73
	ANOVA[a]	N.S.	N.S.	***	**	*
ANOVA[b]	Soil	***	***	***	*	**
	Part	N.S.	N.S.	N.S.	N.S.	N.S.
	Soil × Part	***	***	***	***	**

N.S.: not significant.
[a] One-way.
[b] Two-way.
*, **, ***: significant at $P < 0.05$, 0.01 and 0.001, respectively.

Table 3
Soluble and exchangeable heavy metals ($CaCl_2$ extractable) concentrations ($\mu g\,g^{-1} \pm se$) in the rhizosphere and bulk soil from the rhizopots experiment, and in soil with and without roots at the end of the incubation experiment.

Rhizopots		Cd	Cu	Fe	Mn	Pb	Zn
Soil A	Rhizosphere	0.08 ± 0.01	<0.01	0.61 ± 0.21	0.11 ± 0.05	<0.01	0.80 ± 0.17
	Bulk soil	0.06 ± 0.00	<0.01	0.75 ± 0.19	0.07 ± 0.02	<0.01	0.62 ± 0.08
	ANOVA[a]	N.S.		N.S.	N.S.		N.S.
Soil B	Rhizosphere	0.05 ± 0.00	<0.01	0.76 ± 0.05	0.15 ± 0.01	<0.01	0.32 ± 0.01
	Bulk soil	0.07 ± 0.00	<0.01	0.43 ± 0.02	0.09 ± 0.01	<0.01	0.49 ± 0.05
	ANOVA[a]	**		**	*		N.S.
ANOVA[b]	Soil	N.S.		N.S.	N.S.		*
	Part	N.S.		N.S.	N.S.		N.S.
	Soil × Part	*		N.S.	N.S.		*
Incubation		**Cd**	**Cu**	**Fe**	**Mn**	**Pb**	**Zn**
Soil A	With roots	0.12 ± 0.01	0.15 ± 0.00	1.36 ± 0.18	6.00 ± 0.11	0.18 ± 0.01	0.49 ± 0.13
	No roots	0.14 ± 0.02	0.11 ± 0.00	1.16 ± 0.11	0.17 ± 0.04	0.26 ± 0.02	0.87 ± 0.03
	ANOVA[a]	N.S.	**	N.S.	***	*	***
Soil B	With roots	0.07 ± 0.00	0.06 ± 0.00	0.72 ± 0.04	0.02 ± 0.00	0.15 ± 0.01	0.17 ± 0.01
	No roots	<0.01	<0.01	0.49 ± 0.17	<0.01	0.05 ± 0.02	<0.1
	ANOVA[a]	N.S.	N.S.	N.S	N.S.	*	N.S.
ANOVA[b]	Soil	***	***	**	N.S.	*	***
	Part	N.S.	N.S.	N.S	N.S.	N.S.	N.S.
	Soil × Part	***	***	**	***	**	***

N.S.: not significant.
[a] One-way.
[b] Two-way.
*, **, ***: significant at $P < 0.05$, 0.01 and 0.001, respectively.

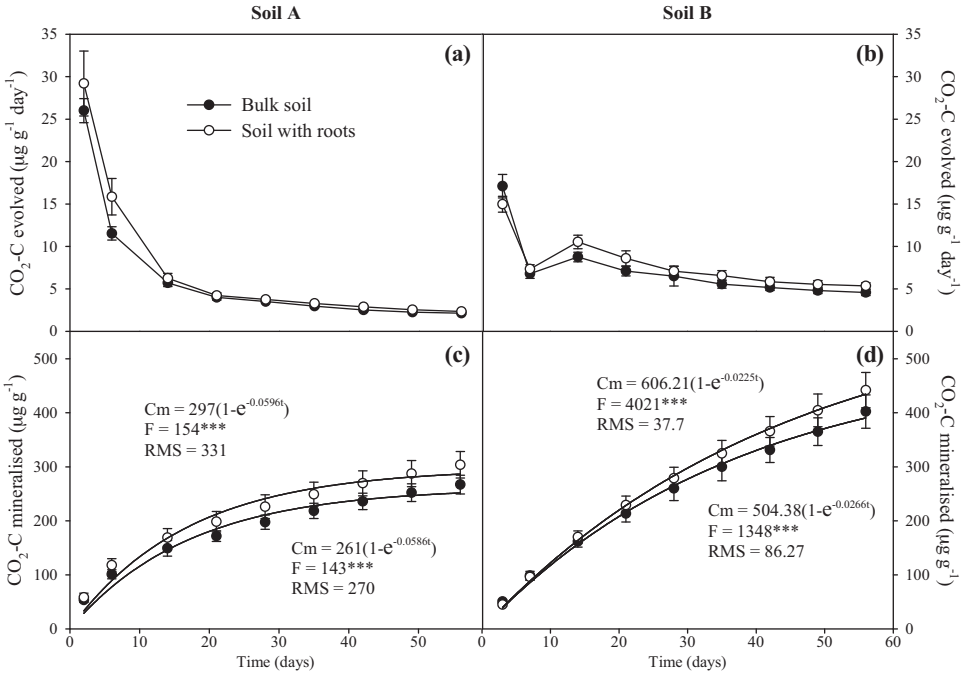

Fig. 3. Rate of CO_2–C evolution during the incubation of control bulk soil and soil with roots ($\mu g\,g^{-1}\,day^{-1}$) (a and b), and organic-C mineralisation of roots in soils after 56 days of incubation—with parameters of the first-order kinetic model, potentially mineralisable-C (C_0), rate constant (k) and statistical parameters of the curve fitting (residual mean square and F-ANOVA) (c and d).

4. Discussion

4.1. Heavy metals availability in soils and their accumulation in *N. caerulescens* plants

The soil heavy metal concentrations did not seem to affect the biomass production of *N. caerulescens*. In agreement with this, McGrath et al. (1997) found similar plant yield (DW) for *N. caerulescens* plants grown for 105 days in three different soils containing moderate to high levels of heavy metals (155–1317 $\mu g\,g^{-1}$ Zn, 80–879 $\mu g\,g^{-1}$ Pb, 2.9–16.5 $\mu g\,g^{-1}$ Cd)—behaviour typical of metallophytes, which are able to develop normally in heavy metal polluted soils. The macronutrient concentrations in the aerial parts were within ranges similar to those reported by Gonneau et al. (2014), for a number of populations of *N. caerulescens*, and slightly higher than those reported for this species in artificially metal enriched soils (Molitor et al., 2005) and in agricultural soils amended with septic tank sludge (Dessureault-Rompré et al., 2010). In any case our values may be considered within the normal range for plants (Marschner, 1995)—with the exception of Mg, the concentrations of which were just above the optimum level (1.5–3.5 $mg\,g^{-1}$; Table A1, SI).

The high sulphate concentrations in the plant tissue extracts could be a consequence of the high sulphur requirement typical of this plant family (Westerman et al., 2001). Several authors (e.g., Dessureault-Rompré et al., 2010) have found low concentrations of sulphate in the rhizosphere of *N. caerulescens*, with respect to the bulk soil, probably due to the high uptake capacity of this species. This plant seems to need several sulphur compounds in order to cope with the heavy metals present in the soil, since Martínez-Alcalá et al. (2013) observed an increase in total thiols, glutathione and cysteine in plants of *N. caerulescens* grown in contaminated soil, related to the elevated concentrations of Cd and Zn in the plants.

The heavy metal concentrations in the plants (Fig. 1) revealed the Cd and Zn accumulator behaviour of *N. caerulescens*. Typical concentrations in the aerial parts of plants are 0.1–10 $\mu g\,g^{-1}$ for Cd and 100–200 $\mu g\,g^{-1}$ for Zn (Milner and Kochian, 2008), although in a previous publication, this species was shown to be able to accumulate up to 10,000 $\mu g\,g^{-1}$ of Cd and 30,000 $\mu g\,g^{-1}$ of Zn in its aerial parts while showing no negative effects (Brown et al., 1995). However, in the present experiment, the plants did not reach the thresholds used to consider this species as a hyperaccumulator of Cd and Zn (100 and 10,000 $\mu g\,g^{-1}$, respectively). This could be due to the presence in the soil of elevated levels of other elements like Cu that restrict Zn accumulation (Walker and Bernal, 2004) and to the characteristics of this ecotype, which does not seem to hyperaccumulate Cd (Walker and Bernal, 2004); therefore, further research with populations able to hyperaccumulate this element would be of interest. Küpper and Kochian (2010) observed a higher concentration of Zn in the aerial parts of *N. caerulescens* when the Zn concentration in the nutrient solution was increased. They also found that Cd addition to the nutrient solution caused a significant decrease of Zn in the aerial parts of the plant. Here, the Fe and Mn concentrations in the aerial parts were lower than those obtained by Molitor et al. (2005), which may be due to the restriction of Fe and Mn uptake by the presence of Cu in the soil (Marschner, 1995; Ebbs and Kochian, 1997; Walker and Bernal, 2004). The multi-elemental contamination of these soils could be, at least in part, responsible for the relatively low Cd and Zn accumulation in the aerial parts of the plant. In fact, the heavy metals concentrations found in this experiment were similar to those obtained in a previous experiment where *N. caerulescens* was grown in similar contaminated soils (Martínez-Alcalá et al., 2013). But, the main limiting factor for hyperaccumulation in these soils was the low availability of the heavy metals in the present experiment, mostly as a consequence of the nearly neutral pH of the soils and the likely formation of iron minerals in the soil due to the pyritic origin of the contamination (Walker et al., 2004).

There is some controversy about the changes that hyperaccumulator plants cause in the properties of the soil directly affected by their roots (Kidd et al., 2009). McGrath et al. (1997) observed a pH decrease in the rhizosphere of *N. caerulescens* (0.2–0.4 units), which could be due to an excess of the assimilation of cations over anions, while Luo et al. (2000) found an increase in rhizosphere pH for *N. caerulescens*. Bernal and McGrath (1994) and Bernal et al. (1994) found increases in the pH of the rhizosphere of the hyperaccumulator *Alyssum murale*, these changes being attributed to the uptake of $N\text{–}NO_3^-$ by the plants. However, changes in soil pH were not observed in the present experiment, so it seems that pH changes in the rhizosphere are not the main limiting factor in the accumulation of heavy metals by hyperaccumulator species (Bernal et al., 1994; Bernal and McGrath, 1994). In fact, Blossfeld et al. (2010) reported alkalinisation in the rhizosphere of *N. caerulescens*, which may decrease both the solubility of metals (Cd) in the soil solution and plant uptake.

Whiting et al. (2001) suggested that microbial biomass and activity play an important role in the bioavailability of Zn to *N. caerulescens*, since microorganisms were able to increase Zn solubility in the soil (from 22 to 67%) and therefore enhance the accumulation of this metal in the aerial parts of the plants. The most relevant effect found in the present experiment concerning microbial biomass (C and N) in the bulk soil and the rhizosphere occurred in the moderately contaminated soil (soil B), where plant root activity enhanced microbial biomass C and N, as well as C_W, indicating improved soil conditions with respect to the bulk soil.

Keller and Hammer (2004) found reduced Cd concentrations in the third crop of *N. caerulescens* when it was used to extract Cd and Zn from contaminated soils. They suggested that replenishment of the bioavailable metal pool in the rhizosphere by the action of the plants had taken place through the displacement of metals from non-rhizosphere sites from the first to the second crop, which was not so effective from the second to the third one. In the present experiment, according to the results of the sequential extraction, Cd and Zn solubility ($CaCl_2$-soluble and EDTA-extractable concentrations) decreased in the rhizosphere of *N. caerulescens*, compared to the bulk soil, which indicates uptake of these elements by the plants and no replenishment of the soluble pool, or replenishment that was slower than the uptake by plants during the growth phase.

Similarly, Gremion et al. (2004) observed that the concentrations of $NaNO_3$-extractable Cd and Zn were significantly lower ($P < 0.05$) in soil sampled from pots planted with *N. caerulescens* than in unplanted pots. Nevertheless, Zhao et al. (2001) found no differences between the root exudates of *N. caerulescens* and those of the non-hyperaccumulator *N. arvense*. Therefore, the higher Cd and Zn accumulation capacity of *N. caerulescens* could be due to its high efficiency in the uptake of these metals and their transport to the aerial parts of the plant and not to a solubilising effect caused by root exudates.

4.2. Effects in soil after plant harvest

Phytoremediation studies dealing with heavy metal contaminated soils have usually focused on the changes in the bio-available pool of heavy metals or their total content in the soil, and on their accumulation by the plants. In our experiment, plant growth and metal accumulation were not high enough to cause any relevant effect on the total metal concentrations in the soil. Nevertheless, the information about the effects of such remediation practices on the soil quality after plant harvest is rather scarce. Once the plants are harvested, root tissues that remain in the soil are degraded by soil microorganisms, at least partially, producing CO_2 (Moreno-Jiménez et al., 2009; Vázquez et al., 2008). In the incubation experiment reported here, the mineralisation of the roots followed a first-order kinetic function, showing a steady decrease of CO_2 evolution during the incubation due to the loss of the biodegradable substrate and the fact that the soil microbial population was large enough to decompose the labile organic compounds from the roots (Vázquez et al., 2008).

The percentage of mineralised-C from *N. caerulescens* roots after 56 days of incubation (7.9–14.4%) was similar to those reported by Moreno-Jiménez et al. (2009), in an experiment with *Rosmarinus officinalis* (13.1%), and by Vázquez et al. (2008) for white lupin (15.8–19.4%)—despite the fact that both these sets of researchers used air–dried, ground roots and not unaltered roots as in the present experiment. During root degradation, metals present in this tissue can be released and incorporated into the soil solution, resulting in increased metal solubility (Perronet et al., 2000). In soil A (Table 3), the $CaCl_2$-extractable Zn concentrations at the end of the incubation experiment were lower in the soil with roots than in the bulk soil (without roots). The latter were slightly higher than those in the rhizopots bulk soil, so there seems to be an incubation effect, which, in any case, does not mean a significant quantitative difference. In fact, in soil B, with a higher pH (Table 2), the opposite effect was observed for Zn (lower concentrations in soils

incubated without roots than in bulk soil), but again no significant differences were found between the soils with and without roots. So, it seems that the high efficiency of *N. caerulescens* with regard to the uptake and transport of Zn caused the fraction of Zn taken up by the plants from the available pool to be clearly higher than the amount released back into the soil solution after root degradation. Also, Walker et al. (2003) indicated that the release of phosphates and other salts during the mineralisation of organic matter may limit Zn and Pb solubility due to the formation of insoluble salts. In the present experiment, the amount of metals released from the roots into the soil during their mineralisation (taking into account the metal concentrations in the roots and the root density in the soil) in both soils was very low (<0.1 $\mu g\, g^{-1}$, with the exception of Fe, which was <2 $\mu g\, g^{-1}$), and the soluble metals fraction in the soil was not significantly increased. Nevertheless, the release of Mn, Pb and Zn into the soil from the roots was lower than for *Lupinus albus* roots in a similar experiment (Martínez-Alcalá et al., 2012).

Boucher et al. (2005) studied the degradation of *Arabidopsis halleri* leaves and Cd and Zn reincorporation to the soil, and observed two main stages. The first involved a fast abiotic transfer of Cd and Zn from readily soluble plant tissues onto fine soil constituents, keeping the metals away from the liquid phase (for about 14 days, the microbial biomass increased as did the metal concentrations in soil). During the second stage (between 14 and 60 days), the Cd and Zn concentrations in the soil solution increased, while the microbial biomass decreased instead of staying constant as it did in the controls (the same soil but with non-contaminated leaves). Perronet et al. (2000) also observed that metals (Cd, Zn) associated with leaves of *N. caerulescens* may be incorporated into the soil after plant death in chemical forms that are available to other plants.

However, the results of the present study show that the amount of heavy metals supplied by the root decay was low (even considering the relatively low root biomass and metal concentrations achieved) and did not alter significantly the solubility of the metals in the soil. So, despite the need to remove the leaves of the hyperaccumulator plants before they wilt, as suggested by different authors (e.g., Perronet et al., 2000), since their metals could be reincorporated into the soil in soluble and/or available forms, it seems that the portion that could be provided by roots is negligible and there is no need to eliminate them from the soil after the phytoremediation process. The behaviour of the metals in soils with higher contamination and metal availability, and/or with populations able to accumulate higher metal concentrations, may need to be evaluated.

5. Conclusions

The efficient uptake and transport of Zn by *N. caerulescens* reduced the soluble and EDTA-extractable Zn concentrations in the rhizosphere, showing its suitability for phytoextraction. Other soil processes, such as precipitation or chelation of heavy metals by root exudates, may have decreased the solubility of the metals. Also, the presence of other metals, like Cu, in the soil could have decreased the efficiency of Cd and Zn extraction due to competition between chemically similar metals.

The reincorporation of heavy metals from degrading roots of *N. caerulescens* into the soils after plant harvest was very low and did not increase overall metals solubility in the soil. Therefore, this particular *N. caerulescens* accession could be successfully employed in the phytoextraction of heavy metals from slightly/moderately polluted soils – by the harvesting and elimination of its aerial parts – although the situation in more severely contaminated soils and with hyperaccumulating populations must be further studied. In any case, the roots can be a source of organic matter for the soil, hence helping to maintain or even enhance its biological activity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ecoleng.2015.11.055.

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