Phytoremediation of mixed-contaminated soil using the hyperaccumulator plant *Alyssum lesbiacum*: Evidence of histidine as a measure of phytoextractable nickel

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Alyssum lesbiacum was shown to phytoextract nickel from PAH-contaminated soils from which the pool of nickel accessed for phytoextraction is closely modelled by a histidine-soil extract.

Abstract

In this study we examine the effects of polycyclic aromatic hydrocarbons (PAHs) on the ability of the hyperaccumulator plant *Alyssum lesbiacum* to phytoextract nickel from co-contaminated soil. Planted and unplanted mesocosms containing the contaminated soils were repeatedly amended with sorbitan trioleate, salicylic acid and histidine in various combinations to enhance the degradation of two PAHs (phenanthrene and chrysene) and increase nickel phytoextraction. Plant growth was negatively affected by PAHs; however, there was no significant effect on the phytoextraction of Ni per unit biomass of shoot. Exogenous histidine did not increase nickel phytoextraction, but the histidine-extractable fraction of soil nickel showed a high correlation with phytoextractable nickel. These results indicate that *Alyssum lesbiacum* might be effective in phytoextracting nickel from marginally PAH-contaminated soils. In addition, we provide evidence for the broader applicability of histidine for quantifying and predicting Ni phytoavailability in soils.

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1. Introduction

Phytoextraction — the transfer of metal from an aqueous or terrestrial source into plant biomass — is one of the few biological approaches for remediating metal-contaminated soils. Metal phytoextraction may be most effectively carried out by metal-hyperaccumulating plants, which specialise in sequestering high concentrations of metal in their above-ground biomass — a trait shared by relatively few species in the world (Baker and Brooks, 1989; Robinson et al., 1997b). A nickel hyperaccumulator is defined as a plant that concentrates nickel

to at least 1000 mg Ni kg⁻¹ in its shoot dry biomass (Brooks et al., 1977). *Alyssum lesbiacum* (Candargy) Rech.f. is among the few Ni hyperaccumulators for which significant progress has been made in detailing the mechanism of metal uptake (Ingle et al., 2005; Kerke and Krämer, 2003). In a number of *Alyssum* species, a strong positive correlation has been found between Ni exposure and histidine production (Krämer et al., 1996). Ingle et al. (2005) demonstrated that the histidine biosynthetic pathway is constitutively more highly expressed in *A. lesbiacum* than in the congeneric non-accumulator species *A. montanum*. The high expression level of histidine biosynthesis genes maintains a large pool of free histidine in the roots, which increases proportionately with Ni uptake (Ingle et al., 2005). Additional species of *Alyssum* are currently being investigated for field-scale metal phytoextraction (Angle et al.,

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2001; Kukier et al., 2004; Li et al., 2003a; Robinson et al., 2003).

Considerable research has demonstrated the contribution of plants to remediation of soils contaminated with organic chemicals through the ‘rhizosphere effect’ – the area around the root where there is an increase in microbial biomass and activity (Arthur et al., 2005; Shaw and Burns, 2003). A wealth of studies have also demonstrated the role of bioaugmentation and biodegradation-enhancing chemicals in facilitating organic pollutant degradation in soil (Singer et al., 2005; van Veen et al., 1997). However, few studies have investigated the ability of metal-hyperaccumulating plants to extract metals effectively in mixed-contaminated soils.

In the present study, we examine the effect of biodegradation-enhancing chemicals and metal chelators on the phytoextraction of Ni in a mixed-contaminated soil. In view of the recent interest in the application of *Alyssum* for metal remediation (Angle et al., 2001; Kukier et al., 2004; Li et al., 2003a,b), *A. lesbiacum* was assessed for Ni phytoextraction in the presence of two polycyclic aromatic hydrocarbons (PAHs), phenanthrene and chrysene. These PAHs are among the most abundant constituents of organic polluted sites (e.g., coal-gas manufacturing plants: ATSDR, 1995). Among the three amendments investigated, two – sorbitan trioleate (surfactant) and salicylic acid (PAH biodegradative inducer) – were selected for their potential contribution towards PAH biodegradation. Sorbitan trioleate, a surfactant containing three C_{16–18} aliphatic groups on a sugar backbone, was applied to enhance the bioavailability of the PAHs, as had been previously demonstrated with polychlorinated biphenyls (Singer et al., 2000) and pentachlorophenol (unpublished data). Salicylic acid, a metabolite of and inducer for the naphthalene operon, was investigated as a soil amendment to stimulate indigenous microbial degradation of PAHs (Chen and Aitken, 1999; Singer et al., 2000). Interestingly, salicylic acid is known to be involved in pathogen-induced defence responses in plants and is present at elevated concentrations in metal-hyperaccumulator plants in the genus *Thlaspi*, where it is implicated in the high degree of cellular tolerance towards nickel (Freeman et al., 2005).

The third amendment, histidine, was selected primarily for its Ni-chelating properties. In lieu of other natural organic-acid chelates such as citrate, oxalate, or acetate (Khodadoust et al., 2004; McGrath and Zhao, 2003), histidine was chosen for its biological compatibility with the presumed mechanism of Ni sequestration in *A. lesbiacum* (Krämer et al., 1996). Its selection also lessens concerns associated with the use of EDTA, a toxic and persistent synthetic chelate employed in previous phytoextraction studies (Meers et al., 2004; Vassil et al., 1998). All soils were evaluated from two depths (0 to 5 cm and 5 to 10 cm) and fractionated into a coarse (>75 µm) and fine fraction (<75 µm) to further examine the distribution and dynamics of Ni within the treatments. Each soil fraction was assayed for the water-, histidine- and acid-extractable Ni pools, representing pools of nickel from which a decreasing percentage of nickel is presumed to be phytoaccessible.

A histidine-utilizing, Ni-tolerant bacterium, which was previously isolated from the experimental soil, was repeatedly

amended to a multi-component treatment to facilitate the disruption of the Ni-histidine complex – a potentially rate-limiting step for the phytoextraction of Ni in *A. lesbiacum*.

In this study, our aims were: (1) to assess the individual and additive effects of salicylic acid, sorbitan trioleate and histidine amendment on the phytoextraction of Ni and biodegradation of PAHs; (2) to examine the relationship between the water-, histidine- and acid-extractable Ni pools and the phytoextractable Ni pool; and (3) to examine the performance of *A. lesbiacum* in co-contaminated (non-serpentine) soil.

2. Materials and methods

2.1. Soil contamination

Thirty kg of uncontaminated agricultural sandy-loam soil (Table 1: Wick series, sandy loam soil from the Wellesbourne National Vegetable Research Station, Warwickshire, UK (Whitfield, 1974)) was air-dried and sieved (2 mm, Mesh No. 10) prior to spiking with Ni and PAHs. Two 10% (w/w) subsamples of the sandy-loam soil were then spiked with either an acetone solution (1:1, v/w) of phenanthrene and chrysene or an acetone-aqueous solution (1:1, v/w) of NiSO₄. The spiked soil subsamples were mixed with Ni, PAH, or pristine soil (1:1) to a final concentration of 100 mg phenanthrene and chrysene kg⁻¹ soil and 50 mg Ni kg⁻¹ soil as described in Section 2.4. The soil Ni concentration of 50 mg kg⁻¹ was chosen: (1) to enable detection of small shifts in the soil Ni pools owing to phytoextraction, since a larger initial soil concentration would have masked these subtle shifts; (2) to assess the potential for using *A. lesbiacum* as a tool to phytoextract metals from marginally contaminated soil; and (3) to evaluate the application of *A. lesbiacum* at the UK government target for residential sites, 50 mg Ni kg⁻¹ soil (Environment Agency, 2002). The soil PAH concentration of 100 mg kg⁻¹ was chosen to provide sufficiently realistic levels of PAH to lend insight into the efficacy of nickel phytoextraction in co-contaminated soils, as well as the sub-lethally toxic response of *A. lesbiacum* to PAHs.

Table 1
Physical and chemical parameters of the experimental soil

	Mean ± SD (n)
Soil chemical parameters	
CEC (cmol kg ⁻¹)	7.71 ± 0.31 (4)
pH	5.74 ± 0.08 (3)
Soil texture ^a	
% Soil particles >75 µm	77 ± 3 (6)
% Soil particles <75 µm	23 ± 3 (6)
% Sand	73 ^b
% Silt	12 ^b
% Clay	14 ^b
Clay	
% Illite	27.0 ± 1.7 (3)
% Expandable ^c	61.7 ± 2.5 (3)
% Kaolinite	11.3 ± 1.2 (3)
Organic matter	
Loss on ignition (%)	2.24 ± 0.01 (3)
% Carbon	0.67 ± 0.01 (3)
% Nitrogen	0.08 ± 0.01 (3)
Carbon:nitrogen	8.37 ± 0.18 (4)
Trace metal (mg kg ⁻¹ soil)	
Ni	24 ± 2.21 (8)

^a Percent soil texture values were determined on a dry mass basis.

^b Whitfield (1974).

^c The ‘expandable’ component in this sample is a random mixed-layer illite/smectite.

The contaminated soils were mixed by rolling within a closed barrel twice weekly for seven weeks to ensure homogeneous contamination and complete removal of solvent vapours. Approximately 0.280 kg of spiked soil was amended to each of 88 experimental columns (see Section 2.4), which were constructed of polyvinylchloride (PVC) pipes (5.08 cm I.D. $\times$ 20 cm length). Columns were initially filled with pristine soil at the bottom (approx. 3 cm) to minimise gas diffusion from beneath the soil profile and to trap leached chemicals, denoted in Fig. 1 as the pristine soil barrier (Singer et al., 2001). The contaminated soil was added to the columns to a depth of 10 cm ($1.43 \text{ g soil cm}^{-3}$) after the addition of a glass-fibre filter barrier to separate the contaminated and pristine soils.

2.2. Planted soil column design

Forty columns were each sown with four *A. lesbiacum* seeds (obtained from Lesvos, Greece, and kindly provided by A.J.M. Baker) on the soil surface and maintained in a glasshouse with natural solar illumination. The soil columns were irrigated daily ($\sim 15 \text{ mL}$ tap water) by an automated overhead sprinkler system for the full length of the study (14 weeks from sowing of seeds). Fourteen days after sowing, columns were restocked with seedlings to compensate for poor germination and mortality, ensuring at least two plants within each planted column at this point. The different amendments were then applied and no further transplants were made, so as to minimize disruption to the soil profile. However, this procedure led to some treatments containing replicate columns with fewer than the desired two plants each (Table 2).

2.3. Bacterial inoculum

A Ni-tolerant histidine utilizing bacterium was previously isolated from the experimental soil by batch enrichment on 3.22 mM L-histidine and 3.22 mM NiSO_4 in minimal salts medium (MSM) (Gilbert and Crowley, 1997) adjusted to pH 7.0 with NaOH. The major species of Ni in solution, as determined by MINEQL+ (Environmental Research Software, Hallowell,

Fig. 1. *Alyssum lesbiacum* and soil profile after removal from PVC pipe. Evidence of dense rooting zone, particularly in the 5 to 10 cm depth.

Table 2
Plant biomass and nickel phytoextraction summary (mean $\pm$ SD, $n = 4$)

Number	Treatment	Focused contrasts ^a	Manipulated ^b	Plants column ⁻¹	Shoot column ⁻¹	Root column ⁻¹	Biomass column ⁻¹	Biomass plant ⁻¹	kg ⁻¹ shoot	kg ⁻¹ root	Shoot Ni plant ⁻¹
Average dry biomass (g)											
1	BaSaSoHis	1 vs. 2	Ba	1.50 ± 0.58	1.11 ± 0.60	0.56 ± 0.46	1.66 ± 1.06	1.11 ± 0.51	1898 ± 523	286 ± 69	1398 ± 325
2	SaSoHis			1.25 ± 0.50	0.63 ± 0.24	0.30 ± 0.13	0.93 ± 0.36	0.74 ± 0.35	1701 ± 287	575 ± 403	861 ± 451
3	SaSoHis-Ni	2 vs. 3	Ni	2.00 ± 1.15	0.73 ± 0.29	0.26 ± 0.12	0.98 ± 0.41	0.49 ± 0.23	100 ± 49	88 ± 55	36 ± 30
4	SaSoHis-PAH	2 vs. 4	PAH	2.00 ± 0.00	1.48 ± 0.31	1.32 ± 1.09	2.79 ± 1.10	1.40 ± 0.55	1583 ± 167	96 ± 105	1167 ± 323
5	SaSo	2 vs. 5	His	2.50 ± 0.58	1.05 ± 0.32	0.63 ± 0.40	1.68 ± 0.66	0.67 ± 0.43	2456 ± 169	127 ± 133	1027 ± 524
6	SaHis	2 vs. 6	So	2.00 ± 0.82	1.10 ± 0.19	0.50 ± 0.21	1.60 ± 0.30	0.80 ± 0.30	1860 ± 602	298 ± 89	1021 ± 711
7	SoHis	2 vs. 7	Sa	2.50 ± 1.29	1.55 ± 0.44	1.01 ± 0.32	2.56 ± 0.66	1.02 ± 0.87	1612 ± 122	163 ± 73	998 ± 803
8	Sa	5 & 6 vs. 8	So/Sa	2.25 ± 0.96	0.83 ± 0.76	0.28 ± 0.35	1.11 ± 1.10	0.49 ± 0.35	2029 ± 633	292 ± 79	751 ± 423
9	So	5 & 7 vs. 9	So/His	2.25 ± 0.50	1.30 ± 0.37	0.67 ± 0.31	1.97 ± 0.66	0.88 ± 0.20	2088 ± 195	264 ± 128	1204 ± 355
10	His	6 & 7 vs. 10	His/Sa	3.25 ± 0.96	1.89 ± 0.72	0.82 ± 0.50	2.71 ± 1.16	0.83 ± 0.31	1659 ± 420	185 ± 135	966 ± 236

Ba = bacterial inoculum, Sa = salicylic acid, So = sorbitan trioleate, His = histidine. All treatments contained Ni and PAH except those denoted by -Ni = without Ni, or -PAH = without PAH.

^a Focused contrasts denote *a priori* hypothesis about treatment effects; treatment numbers in the first column are used to denote treatment contrasts being made.

^b 'Manipulated' denotes the treatment variables that are highlighted in the focused contrast.

ME, USA: Schecher and McAvoy, 1992) and GEOCHEM-PC, version 2.0 (Parker et al., 1995), were Ni(His) and Ni(His)₂, where 99% of the histidine was complexed with Ni. The bacterium was selected from other isolates because of its rapid growth. A 398 kb fragment from the 16S rRNA of this isolate gave a 99% nucleotide sequence identity to *Arthrobacter histidinovorans* (data not shown). Previous reports of this bacterium indicate its ability to metabolise histidine as well as convert histidinol to histidine (Adams, 1954). It was hypothesized that repeated addition of this isolate to planted soils would expedite dissociation of the histidine-Ni complex, and possibly increase phytoextraction, if dissociation was necessary for transport of Ni into the root of *A. lesbiacum*.

2.4. Experiment design and analysis

Four replicates of ten planted and unplanted treatments were amended with one or more of the following treatments, as per Table 2: *A. histidinovorans* (Ba), 3.6 mM salicylic acid (Sa), 0.8 mM sorbitan trioleate (So), and 3.22 mM L-histidine (His). Treatments were repeatedly added in 15 mL aliquots twice weekly. Two additional unplanted treatments were prepared: (1) co-contaminated soil amended with minimal salts medium (MSM); and (2) a pristine soil control (Pristine). *A. histidinovorans* was prepared for treatment BaSaSoHis by culturing the bacterium in a 250 mL Erlenmeyer flask containing 100 mL of MSM and 3.22 mM His. The culture was combined with 100 mL of MSM containing 7.2 mM Sa, and 1.6 mM So prior to addition to the soil, to make a final amendment concentration of 3.6 mM Sa and 0.8 mM So.

2.5. Sampling and chemical analysis

The soil columns were dismantled 12 weeks after the first addition of amendments (14 weeks from sowing), by carefully separating the 0 to 5 cm and 5 to 10 cm depths and pristine soil barrier (Fig. 1). Using an ethanol-washed stainless steel spatula, each depth was mixed thoroughly and dispensed into 40-mL borosilicate vials before being frozen at -80°C and freeze-dried. Each of the four treatment replicates at both depths were sieved (200 mesh, 75 μm) into a fine and coarse fraction. These sieved soil fractions were then subjected to water-, histidine- and nitric acid extractions as described below.

Water-extractable Ni analyses were carried out on 1.00 g soil in 50-mL polypropylene centrifuge tubes containing 9.0 mL of 0.1 M MgCl_2 solution, while histidine-extractable Ni analyses were carried out similarly in a 3.22 mM L-histidine solution (pH 7.0). In both cases, samples were shaken horizontally for 60 min, followed by centrifuging at $3000 \times g$ for 15 min (20°C). The supernatant was passed through a 0.45 μm PTFE syringe filter into a 15 mL polypropylene tube for analysis by atomic absorption spectrophotometry (AAS; wavelength: 232.0 nm, flame gases: air and acetylene, detection limit: 0.02 mg L^{-1} , sensitivity: 0.15 mg L^{-1} , optimum range: 0.3 to 10 mg L^{-1}) using an AAAnalyst 100 with deuterium arc background correction (Perkin-Elmer, Beaconsfield, Buckinghamshire, UK). Acid-extractable Ni extractions were carried out on 100 mg soil (dry mass), to which 3 mL nitric acid (69%, v/v) was added. The sample was vortexed for 1 min, and left incubating at 70°C for 36 h. After incubation, 4 mL of deionized water was added to each vial, vortexed, centrifuged at $3000 \times g$ for 15 min (20°C) and passed through a 0.45 μm PTFE filter for analysis by AAS. The nitric-acid extractable Ni procedure was adopted in lieu of an aqua regia digest, as there was no significant difference in extractable nickel between the two methods for the experimental soil (data not shown).

Phenanthrene and chrysene were extracted from 1.00 g soil in duplicate from unfractionated soil at each of the depths. The soil was vortexed for 1 min in 4 mL dichloromethane:acetone (3:1), followed by a 2 h incubation in a sonicating bath (34°C) and agitation for 18 h on a horizontal shaker. The selection and ratio of PAH extraction solvents were experimentally determined to yield the greatest PAH recovery from the experimental soil (data not shown). Analysis of PAH extractions were performed by gas chromatography-mass spectrometry (Perkin-Elmer Autosystem GC-MS), equipped with an Agilent DB-5 ms capillary column (30 m length $\times$ 0.25 μm film thickness $\times$ 0.25 mm ID). A 1 μL injection was analysed with the following instrument parameters: injector temperature 280°C , detector and transfer line 230°C ; He carrier at 17 psi and 40 mL min^{-1} split. The temperature program

initiated at 60°C , held 1 min, ramped $15^{\circ}\text{C min}^{-1}$ to a final 270°C , and held 12 min. Quantification was based on single-ion monitoring of biphenyl (internal standard) at m/z 155, phenanthrene at m/z 178, and chrysene at m/z 228.

2.6. Plant analysis

Plant roots were carefully removed from the soil, agitated for approximately 1 min in 10 mM EDTA, blotted dry and weighed. The root and shoot samples were subsequently stored in a paper bag at -20°C until Ni analysis. Plant material was oven-dried (70°C), crushed into a powder, and prepared in duplicate for microwave digests by adding 5 mL of 69% (v/v) HNO_3 into an extraction vial containing 250 mg of plant material. Samples were digested in CEM advanced composite pressure vessels using a CEM MDS 2000 microwave digestion system (CEM Microwave Technology, Buckingham, UK) and then analysed by flame AAS as described above.

2.7. Soil analysis

Percent carbon and nitrogen was determined in the 5 to 10 cm depth, in duplicate, using a Europa Roboprep/VG 622 mass spectrometer (Europa Scientific Ltd, Cheshire, UK). Using the reference atropine (4.84% nitrogen and 70.5% carbon) and the certified reference GBW 07412, IAEA-309, and LECO standard calcium carbonate for quality control (Table 3). Soil pH was measured using a digital pH meter from 1.00 g of soil from both soil depths after vortexing in 0.1 M MgCl_2 and centrifuging at $3000 \times g$ for 5 min at 20°C (Table 3).

2.8. Chelating study

A small study was conducted to quantify the effects of each amendment, singularly and in combination, on Ni desorption. Triplicate 1.00 g freshly-spiked experimental soil aliquots containing 200 $\text{mg NiSO}_4 \text{ kg}^{-1}$ soil were extracted with 15 mL of a 3.22 mM solution of each of the following amendment combinations in deionised water (pH adjusted to 7.0 with NaOH or HNO_3): (1) $\text{Ca}(\text{NO}_3)_2$; (2) Sa; (3) So; (4) His; (5) SaSoHis; (6) EDTA; and (7) SaSoHis-EDTA. Samples were prepared and analysed by AAS as described in Section 2.5.

2.9. Statistics

As the unbalanced experimental design precluded the use of conventional analysis of variance, we chose to conduct focused contrasts of particular amendment combinations (Rosenthal and Rosnow, 1985) based on our *a priori* hypotheses on how particular amendments would affect the response variables. Significance is presented as a result of these focused contrasts unless otherwise stated. We also used the Tukey HSD test for particular cases when we were interested in comparing all the amendment combinations against each other. Most statistical analyses were conducted using R version 1.9.0, while paired two sample for means *t*-tests were conducted on Microsoft Excel 2002. Unless otherwise specified, statistical significance was at the $P < 0.05$ level, while errors ($\pm$) of presented data are standard deviations.

3. Results and discussion

3.1. Chelating study

In the chelating study (Fig. 2), we examined the effects of chemical amendments on Ni desorption in freshly spiked Ni-contaminated soil (200 mg Ni kg^{-1}). In the control treatment (3.22 mM $\text{Ca}(\text{NO}_3)_2$), $13 \pm 1\%$ of the total soil Ni was desorbed. Neither Sa nor So desorbed greater Ni than the control extractant ($P > 0.05$), suggesting a limited role for these amendments in Ni desorption for this study. Khodadoust

Table 3
Distribution of nickel by depth and extractant in water-, histidine- and acid-extractable soil, either planted and unplanted (expressed as mg Ni kg⁻¹ soil)

Treatment	0 to 5 cm depth			5 to 10 cm depth			Leachate depth		
	Water	Histidine	Acid	Water	Histidine	Acid	Water	Histidine	Acid
Planted									
BaSaSoHi	3.7	8.5	46.6	2.1	5.1	34.9	0.0	0.5	10.5
SaSoHi	3.8	8.6	43.3	3.2	6.9	42.7	0.1	0.3	12.1
SaSoHi-Ni	0.0	0.5	27.2	0.0	0.2	22.7	0.0	0.2	10.9
SaSoHi-PAH	3.9	5.2	44.6	1.8	4.2	40.4	0.0	0.5	10.4
SaSo	3.1	5.2	38.4	1.2	3.8	37.7	1.0	2.5	10.5
SaHi	3.0	7.6	35.4	2.4	5.9	37.9	0.1	0.7	10.0
SoHi	3.0	7.2	44.5	2.1	5.9	35.9	0.0	0.6	9.9
Sa	1.8	6.3	45.3	1.9	5.8	40.6	0.1	0.4	13.6
So	2.3	6.1	40.7	1.1	4.9	40.3	0.0	0.4	12.1
Hi	2.1	6.8	38.8	1.5	5.1	35.6	0.0	0.4	10.8
Unplanted									
BaSaSoHi	3.7	8.4	41.7	2.2	6.2	46.1	0.5	1.5	10.3
SaSoHi	3.9	7.9	52.8	5.1	8.1	44.2	0.1	0.9	12.4
SaSoHi-Ni	0.0	0.2	21.7	0.1	0.3	21.7	0.0	0.2	10.4
SaSoHi-PAH	4.6	9.8	43.5	3.7	8.1	43.2	0.1	0.5	13.6
SaSo	4.4	8.6	49.2	3.5	6.9	47.2	0.1	0.6	13.7
SaHi	5.2	8.5	44.5	4.8	7.6	44.3	0.1	0.9	10.4
SoHi	5.1	7.5	43.5	3.2	7.1	44.7	0.1	0.7	11.3
Sa	4.9	7.1	43.8	3.2	7.1	44.2	0.0	0.6	11.7
So	4.8	8.3	44.4	4.1	8.0	43.4	0.0	0.8	12.5
Hi	4.4	8.0	39.7	4.2	7.9	44.3	0.1	0.7	10.7
MSM	5.1	8.4	45.4	3.7	7.3	44.0	0.0	0.6	10.9
Pristine	0.1	0.5	22.7	0.1	0.2	23.6	0.0	0.2	10.9

There are 140 g of soil for each of the two depths 0 to 5 cm and 5 to 10 cm.

et al. (2004) also found marginal desorption of soil Ni (10 to 16%) using Tween 80, a C₁₈ sorbitol-based surfactant similar to sorbitan trioleate. In contrast, histidine mobilised nearly 50% of the soil Ni (98 ± 8 mg Ni kg⁻¹ soil), which was significantly greater than the control ($P = 0.006$) and similar to the EDTA extract, thus confirming the efficacy of histidine as a strong Ni-binding ligand. No significant increase in Ni desorption was achieved with the combined addition of SaSoHi or SaSoHisEDTA as compared to histidine alone (Fig. 2).

3.2. Treatment effects

Alyssum lesbiacum, a well-characterised Ni-hyperaccumulating plant, was grown in Ni and PAH co-contaminated soil that was repeatedly amended with different treatments to test individual and combined treatment effects on the phytoextraction of Ni. At the conclusion of the study (14 weeks), treatment SaSoHis-PAH had significantly greater total plant biomass per column ($P = 0.0029$) as compared to treatment SaSoHis (Table 2), indicating a degree of PAH toxicity in *A. lesbiacum*. Treatment SaSoHis also contained 600% more Ni per unit mass of root than the corresponding treatment SaSoHis-PAH, which might lend some insight into the mechanism of PAH toxicity. Toxicity was most apparent within the first two weeks, when poor seed germination and greater mortality was observed (data not shown). Owing to PAH biodegradation and aging during the study, PAH phytoavailability would have declined rapidly in the early weeks with a concomitant decline in phytotoxicity (White et al., 1998), thereby facilitating the recovery of plant health in PAH-containing treatments. Although toxic effects of PAHs on plant biomass were observed early in the study, this did not significantly impinge on the phytoextraction of Ni per unit mass of shoot (Table 2).

Greater than 90% of the initial soil PAH spike biodegraded within the 14 week study irrespective of amendment or presence of plant (data not shown). The extensive PAH removal in this study can be attributed to a number of factors, most notably low soil organic matter (2.24%) and high coarse soil fraction (77%), which confer low PAH sorption and high bioavailability

Fig. 2. Nickel desorbed, as a percentage of total nickel spike, with the singular and combined addition of 3.22 mM of the different amendments: salicylic acid (Sa), sorbitan trioleate (So), histidine (His). EDTA was included for comparison. Initial soil nickel spike was 200 mg kg⁻¹ NiSO₄. Treatments denoted by different lower-case letters are significantly different ($P < 0.05$).

(Essington, 2004). The similarity between phenanthrene and chrysene chemical structures might have hastened PAH degradation as a single enzyme might be capable of degrading both chemicals. Repeated addition of readily degradable carbon substrates (sorbitan trioleate, salicylic acid and histidine), might provide sufficient nutrients for indigenous microbial population to proliferate and biodegrade the PAHs.

Nickel accumulation in the plant shoot was not enhanced by the exogenous provision of histidine to the soil ($P > 0.05$, Table 2: focused contrast '2 vs 5' and '6 & 7 vs. 10'). This lack of effect might reflect the naturally high concentrations of endogenous histidine in *A. lesbiacum*, and is consistent with previous research showing no significant increase in Ni flux into the xylem upon addition of histidine into the rooting medium of hydroponically grown *A. lesbiacum* (Kerkeb and Krämer, 2003; Krämer et al., 1996). The insensitivity of Ni flux in *A. lesbiacum* to exogenously supplied histidine contrasts with that of non-hyperaccumulating plants, such as *A. montanum* and *Brassica juncea*, which show enhanced Ni flux into the xylem upon addition of exogenous L-histidine in a hydroponic rooting medium (Kerkeb and Krämer, 2003; Krämer et al., 1996). *B. juncea* was also shown to be capable of accumulating up to 1.1% (w/w) of lead in its shoot tissue dry biomass when exposed to a hydroponic solution of Pb and EDTA (Vassil et al., 1998). *Zea mays* also demonstrated a 140-fold increase in lead translocation in the xylem sap after application of an EDTA solution to the soil (Huang et al., 1997). However, unlike hyperaccumulating species, which exhibit a naturally high tolerance to metal concentrations in their shoot biomass of 1% (w/w) or greater, non-hyperaccumulator plants rapidly succumb to metal toxicity upon accumulating tissue metal concentrations of this magnitude, necessitating their harvest to ensure that phytoextracted metal is not released onto site through leaf senescence and shedding. The addition of *Arthrobacter histidinolovorans* in treatment BaSaSoHis did not appear to influence the extent of phytoextracted Ni per unit biomass of shoot, suggesting that the transfer of Ni from the soil solution into the plant was not a limiting step under these experimental conditions. As the inoculum was repeatedly amended, we do not think its poor survival in the soil precluded any potential effect.

3.3. Dynamics of the soil nickel pools

Soils from both the 0 to 5 cm and 5 to 10 cm depths were prepared for Ni analysis by fractionation into coarse ($>75 \mu\text{m}$) and fine ($<75 \mu\text{m}$) fractions, accounting for approximately 77% and 23% of the total soil by mass, respectively. Each soil fraction was assayed for the water-, histidine- and acid-extractable Ni pools, representing pools of nickel from which a progressively smaller percentage of nickel is presumed to be phytoaccessible (Table 3). Both the fine and coarse fractions of the unspiked study soil contained negligible amounts of water- and histidine-extractable Ni, with $18.40 \pm 2.23 \text{ mg Ni kg}^{-1}$ of the $23.15 \pm 2.21 \text{ mg Ni kg}^{-1}$ unfractionated soil residing on the coarse fraction. However, roughly all of the Ni spiked into the soil at the outset of the study

partitioned into the fine fraction, increasing its acid-extractable Ni concentration from $20.65 \pm 0.9 \text{ mg Ni kg}^{-1}$ to $120.95 \pm 5.35 \text{ mg Ni kg}^{-1}$ soil (MSM treatment; $P < 0.0001$).

3.4. Phytoextracted nickel pool

On average, $2297 \pm 955 \mu\text{g Ni}$ was phytoextracted into the plant biomass per column, accounting for approximately $19 \pm 9\%$ of the average acid-extractable Ni from the corresponding unplanted control soil (Table 2; $12520 \pm 1239 \mu\text{g Ni}$ per column). The average percentage Ni recovery in the plant biomass compares favourably with the results of the study of Li et al. (2003b), in which two other hyperaccumulator species of *Alyssum*, *A. murale* and *A. corsicum*, were shown to phytoextract 11% of total acid-extractable Ni from Quarry organic soil. Greater percentage recovery of Ni in this study is likely due to the modest Ni contamination coupled with the high efficiency of *A. lesbiacum* in phytoextracting Ni from marginally contaminated soils.

The pool of Ni that is solely histidine-extractable (E_{h-w}) can be defined by subtracting the water-extractable nickel fraction (E_w) from the total histidine-extractable nickel (E_h ; Table 3). Within the 0 to 5 cm depth, the average E_{h-w} pool, 207 mg Ni, was identical in size for both planted and unplanted soils. In the 5 to 10 cm depth, there was $25 \mu\text{g}$ less Ni recovered from the planted soil ($189 \mu\text{g Ni/depth}$) than the unplanted ($214 \mu\text{g Ni/depth}$). The E_{h-w} pool in the 5 to 10 cm depth might have been depleted relative to the 0 to 5 cm depth, as it represents the region with the highest root density (qualitatively assessed, data not shown).

3.5. Indicator of phytoextractable nickel

The ratio (E_r) of E_{w*} , E_{h*} and acid-extractable nickel (E_{a*}) to the phytoextractable Ni (E_p), provides insight into the phytoaccessibility of each of the Ni pools, where the asterisk denotes values from the respective unplanted treatments. The respective unplanted soils were used as a reference for this calculation to account for non-plant treatment effects on Ni availability. Hence, E_r should equal unity (1.0) when an extractant removes precisely the 'phytoaccessible' Ni pool.

The average E_r for water-extractable nickel suggests that E_{w*} greatly underestimates the E_p , with an E_r of 2.44 ± 0.95 (Fig. 3A), indicating that the average planted soil has at least twice as much Ni in the plant biomass as can be provided by E_{w*} . E_{h*} very closely approximates E_p , with an average E_r of 1.14 ± 0.43 (Fig. 3A). E_r was slightly greater than unity in a few treatments (i.e., SaSo, SoHis, So, His; Fig. 3B), which might be explained by the following: (1) the plant roots had extended into the pristine soil barrier, enabling the plants to access the additional 2 mg Ni estimated to be in the pristine soil barrier; and (2) E_p was acquired over a 12 week period from a dynamic, not static, pool of phytoaccessible nickel. A dynamic equilibrium between nickel pools is maintained during the 12 week study as the plant phytoextracts nickel, which is difficult to estimate using an instantaneous method of extracting nickel. Nonetheless, histidine provides a good

Fig. 3. (A) Average of extraction ratios (E_r) from all treatments: phytoextracted nickel (E_p) to water- (E_w), histidine- (E_h) and acid- (E_a) extractable nickel. (B) Average E_r for each treatment, where black, shaded and white columns indicate the E_r for E_w , E_h and E_a , respectively.

approximation of the phytoextractable pool for *A. lesbiacum*. As anticipated, E_{a*} greatly overestimated E_p , with an E_r of 0.19 ± 0.07 , demonstrating that *A. lesbiacum* had phytoextracted approximately 20% of E_{a*} (Fig. 3A). In general, E_p fell within the range achieved in the study of Li et al. (2003b), in which *A. murale* phytoextracted upwards of 39% of the total DTPA-extractable Ni. However, as the authors used DTPA (diethylenetriaminepentaacetate)-extractable nickel as a benchmark for phytoaccessible nickel, and employed a different soil and plant in their study than used in the present work, it is not possible to compare these two studies critically.

3.5.1. Soil pH effects

Sulfur and organic acids have previously been shown to enhance metal phytoextraction, possibly in part by lowering soil pH, as well as by ligand-promoted desorption of metals from fixed exchange sites (Kayser et al., 2000; Meers et al., 2004; Robinson et al., 1997a, 1999). However, Li et al. (2003b) observed an increase in Ni uptake by two *Alyssum* spp. (*A. murale* and *A. corsicum*) as pH increased. The authors offered several explanations for this: (1) the plant is better adapted to the phytoextraction of Ni at higher soil pH; (2) less competition with divalent cations for Ni uptake at higher pH; (3) increased

phytoavailability of organically bound Ni at higher pH; (4) low binding of Ni to the soil minerals; and (5) more effective binding and uptake of Ni with histidine at higher soil pH. In our study, the pH of planted soils was significantly higher than the corresponding unplanted treatments, with average pH values of 5.61 ± 0.30 and 5.75 ± 0.26 in planted soils, and 5.19 ± 0.27 and 5.45 ± 0.23 in unplanted soils, at 0 to 5 cm and 5 to 10 cm depths, respectively ($P < 0.05$; Table 4). In this study, there was a weak, yet significant, positive correlation between phytoextracted Ni per column and soil pH (5 to 10 cm depth; $R^2 = 0.168$; $F = 7.69$, $P = 0.0085$, $n = 36$), thereby lending some support to the findings and hypothetical mechanisms proposed by Li et al. (2003b).

3.5.2. Bioconcentration factor

In the present study, Ni bioconcentration (mg Ni kg^{-1} shoot dry biomass/ mg Ni kg^{-1} acid-extracted unfractionated soil) averaged 50 ± 13 , which compares positively with an average bioconcentration factor of 60 observed in *A. bertolonii* growing in natural serpentine soils (Robinson et al., 1997b). Interestingly, the bioconcentration factors were similar between the two investigations, despite the soil used in the *A. bertolonii* study containing a Ni concentration ($1550 \text{ mg Ni kg}^{-1}$ soil) 32 times greater than in the present study.

4. Conclusions

As evident in our study, the hyperaccumulator plant *Alyssum lesbiacum* can be very effective in phytoextracting Ni

Table 4

Means of soil pH by depth (mean $\pm$ SD, $n = 4$) and carbon:nitrogen ratio ($n = 2$)

		0 to 5 cm		5 to 10 cm
		pH	pH	C:N
(%C/%N)				
Planted	BaSaSoHis	5.40 ± 0.21	5.59 ± 0.22	11.2
	SaSoHis	5.46 ± 0.28	5.43 ± 0.14	9.4
	SaSoHis-Ni	5.40 ± 0.13	5.63 ± 0.17	11.1
	SaSoHis-PAH	5.52 ± 0.09	5.78 ± 0.16	9.3
	SaSo	5.64 ± 0.18	5.88 ± 0.17	10.1
	SaHis	5.52 ± 0.26	5.57 ± 0.19	9.7
	SoHis	5.58 ± 0.32	5.91 ± 0.17	10.1
	Sa	6.15 ± 0.20	5.91 ± 0.24	9.7
	So	5.93 ± 0.21	6.12 ± 0.22	11.2
	His	5.54 ± 0.32	5.68 ± 0.13	10.7
Unplanted	BaSaSoHis	5.15 ± 0.06	5.53 ± 0.12	11.4
	SaSoHis	5.06 ± 0.06	5.23 ± 0.06	11.5
	SaSoHis-Ni	4.95 ± 0.10	5.27 ± 0.18	11.2
	SaSoHis-PAH	5.26 ± 0.13	5.5 ± 0.09	9.7
	SaSo	5.50 ± 0.07	5.64 ± 0.06	9.5
	SaHis	5.10 ± 0.14	5.25 ± 0.11	8.4
	SoHis	4.80 ± 0.07	5.17 ± 0.09	8.4
	Sa	4.99 ± 0.22	5.51 ± 0.11	8.3
	So	5.54 ± 0.05	5.71 ± 0.24	10.0
	His	5.53 ± 0.07	5.74 ± 0.09	8.7
	MSM	5.19 ± 0.11	5.39 ± 0.08	8.6
	Pristine	5.25 ± 0.10	5.74 ± 0.22	8.2

from modestly contaminated soils, as well as from soil co-contaminated with phenanthrene and chrysene. Moreover, there was no significant decline in the concentration of nickel in the shoot of *A. lesbiacum* in the presence of PAHs, suggesting that, within limits, *A. lesbiacum* might be effective in treating mixed-contaminated soils containing PAHs. This study provides the first demonstration of the application of histidine as an indicator of phytoextractable Ni from soils. The close agreement between nickel extractability using histidine and nickel phytoaccessibility might have broader implications, since inexpensive assays for the determination of pollutant bioavailability are critically needed for risk assessment, cost-benefit analyses and selection of appropriate remediation strategies for contaminated soils.

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