

PLANT-MICROBE-SOIL INTERACTIONS AND THEIR ROLE IN PHYTOTECHNOLOGIES APPLIED TO TRACE METAL-RICH SOILS

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Plant-microbe-soil interactions and their role in phytotechnologies applied to trace metal-rich soils

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INFORMAN:

Que la presente memoria titulada "Plant-microbe-soil interactions and their role in phytotechnologies applied to trace metal-rich soils", presentada por Dña Vanessa Álvarez López para optar al Grado de Doctora en Biología, fue realizada bajo nuestra dirección.

Y considerando que representa trabajo de Tesis de Doctorado, autorizamos su presentación ante el Tribunal correspondiente.

Y para que así conste, firmamos el presente informe en Santiago de Compostela a 1 de junio de 2016.

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Table of Contents

Resumen **i**

Introducción. ... iii
Objetivos y principales tareas realizadas ... x
Principales resultados y conclusiones ... xii
Referencias ... xxii

1. Introduction

1.1. Trace elements in soils ... 3
Natural metal(loid)-enriched soils ... 4
Metal-hyperaccumulating plants ... 5
Trace metal contamination in soils ... 8
1.2. Soil trace element availability and plant uptake and accumulation ... 11
Metal(loid) availability in the rhizosphere of hyperaccumulators ... 13
Estimating soil metal(loid) availability to plants ... 16
DIFS (DGT induced fluxes in sediments and soils) ... 20
1.3. Gentle remediation options targeting trace element-contaminated land ... 22
1.4. Improving efficiency of gentle remediation options ... 24
Selection of adequate plant species for implementing gentle remediation options ... 24
Soil amendments and fertilisation regimes for modifying trace element availability and optimising plant growth ... 32
Plant cropping patterns to improve gentle remediation options performance ... 40
The role of plant associated-bacteria in gentle remediation options effectiveness ... 43
1.5 References ... 53

2. Objectives **79**

3. Rhizobacterial communities associated with the flora of three serpentine outcrops of the Iberian Peninsula **85**

3.1. Introduction ... 87
3.2. Materials and methods ... 88
Study areas and collection of samples ... 88
Elemental analyses of field-collected soils and plant material ... 90
Microbiological analyses ... 90
Phenotypic characterisation of bacterial isolates ... 91
Genotypic characterisation of bacterial isolates ... 92

BOX-PCR genomic DNA profiling 92
DNA extraction and 16S rRNA gene amplification 93
Statistical analyses 94
3.3. Results 94
General soil physicochemical properties 94
Culturable bacterial densities and the rhizosphere effect 94
Nickel resistance of the culturable bacterial community 96
Phenotypic characteristics of the bacterial isolates 98
Identification and genotypic diversity of culturable bacteria 103
BOX-PCR profiles 103
16S rDNA identification 114
3.4. Discussion 122
3.5. References 128

4. Nickel availability in the rhizosphere of the Ni-hyperaccmulator *Alyssum pintodasilvae* assessed via conventional soil analyses and diffusive gradient in thin films (DGT) 135

4.1. Introduction 137
4.2. Materials and Methods 139
Study soil and plant species 139
Plant and soil analyses 140
Diffusive Gradient in Thin Films (DGT) measurements 141
DIFS modelling 143
High-resolution Ni fluxes along plant roots 146
4.3. Results 147
Soil physicochemical properties and plant ionome 147
Soil Ni availability and fractionation 148
Soil Ni replenishment kinetics 154
Chemical Imaging of DGT-available Ni along the root 157
4.4. Discussion 158
4.5. Conclusions 164
4.6. References 164

5. Organic amendments for improving biomass production and metal yield of Ni-hyperaccumulating plants 171

5.1. Introduction 173
5.2. Materials and Methods 174
Plant species, study soil and experimental set-up 176
Plant and soil analyses 177
Statistical analyses 177

5.3. Results 177
Effect of fertilisation and compost amendments on soil physico-chemical properties 177
Plant biomass production, shoot Ni concentration, Ni yield and bio-concentration factor (BCF) in untreated soils 179
Effect of fertilisation and compost amendments on plant biomass production 180
Effect of fertilisation and compost amendments on plant nutritive status 181
Effect of fertilisation and compost amendments on soil Ni availability, shoot Ni concentration, Ni yield and bioconcentration factor (BCF) 183
5.4. Discussion 186
5.5. Conclusions 192
5.6. References 193

6. Evaluating phytoextraction efficiency of two high-biomass crops after soil amendment and inoculation with rhizobacterial strains 197

6.1. Introduction 199
6.2. Materials and Methods 201
Mine-soil, plant species and rhizobacterial strains selected for the pot experiment 201
Experimental set-up and inoculation of plants 202
Chlorophyll fluorescence 203
Leaf spectral reflectance 203
Plant and soil analyses 204
Statistical analyses 205
6.2. Results 205
Effect of compost on soil properties and metal availability 205
Plant growth and metal accumulation in untreated and compost–amended soils 207
Effects of rhizobacterial strains on biomass production, plant physiological status and shoot ionome 209
Effects of bacterial inoculants on soil metal availability and fractionation 213
Effects of rhizobacterial inoculants on shoot metal concentrations and metal yield of *Nicotiana tabacum* and *Salix caprea* 217
6.4. Discussion 220
6.5. Conclusions 225
6.6. References 226

7. Inoculation methods using *Rhodococcus erythropolis* strain P30 affects bacterial assited phytoextraction capacity of *Nicotiana tabacum* 233

7.1. Introduction 235
7.2. Materials and Methods 236
Soil and plant species selected for the pot experiment 236
Rhizobacterial strain and inoculation treatments 237
Plant and soil analyses 239
Statistical analyses 239
7.3. Results 240
Effect of bacterial inoculation on biomass production 240
Shoot metal concentrations and effect of bacterial inoculation on shoot ionome and metal accumulation 242
Effect of bacterial inoculation on metal availability in soils 245
Effect of bacterial inoculation on plant metal yield 246
7.4. Discussion 247
7.5. Conclusions 251
7.6. References 251

8. Monitoring the effectiveness of (aided) phytostabilisation implemented in Cu-rich mine tailings) 257

8.1. Introduction 259
8.2. Materialsand Methods 261
Study site 261
Experimental design and set-up 261
Soil sampling and analyses 262
Monitoring of plant survival, growth and ionome 264
Statistical analyses 265
8.3. Results 265
Effect of compost addition on soil physico- and bio-chemical properties 265
Evolution in plant growth and shoot ionome 269
Effects of plant species on soil physico- and bio-chemical properties 274
8.4. Discussion 278
8.5. Conclusions 284
8.6. References 285

9. Implementation of (aided) phytoextraction in Pb/Zn mine tailings using hyperaccumulating and high biomass-producing plant species 291

9.1. Introduction 293
9.2. Materials and Methods 295

Study site 295
Experimental design and set-up 296
Soil sampling and analyses 297
Monitoring plant survival, growth and ionome 301
Statistical analyses 302
9.3. Results 302
Effect of compost addition on soil physico- and bio-chemical properties 302
Effects of plant growth on soil physico- and bio-chemical properties 307
SRC of *Salix smithiana* and *Salix atrocinerea* 307
Cultivation of the hyperaccumulator *Noccaea caerulescens* 310
Plant growth, shoot ionome and metal yields 314
SRC of *Salix smithiana* and *Salix atrocinerea* 314
Cultivation of the hyperaccumulator *Noccaea caerulescens* 322
9.4. Discussion 330
9.5. Conclusions 341
9.6. References 342

10. Final synthesis and conclusions **353**

Agradecementos **359**

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RESUMEN

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INTRODUCCIÓN

El término elemento traza se refiere a aquellos elementos que se encuentran en concentraciones menores a 0.1% (1000 mg kg^{-1}) en el medio ambiente o menores a 0.01% (100 mg kg^{-1}) en tejidos animales y vegetales (Adriano, 2001). Las denominaciones metales traza (que hace referencia a su abundancia y que incluye metales y metaloides) o metales pesados (que indica que sus densidades son > 5 g cm^{-3}) también se usan con frecuencia para estos elementos y se consideran sinónimos (Kabata-Pendias, 2010). Algunos metales traza (como el Cu, Fe, Mn, Ni o Zn) son micronutrientes esenciales y son imprescindibles en determinados procesos celulares; por el contrario, a otros como As, Cd, Hg o Pb no se les conoce ninguna función biológica y se consideran no-esenciales. En ambos casos, un exceso de estos elementos afecta severamente a procesos biológicos en organismos vivos lo que causa un riesgo importante para el medio ambiente y la salud humana (Cuypers *et al*., 2010).

Suelos naturalmente ricos en metales

Sin tener en cuenta la actuación del ser humano, la concentración de elementos traza en los suelos depende del material de partida y de sus procesos de meteorización. Los tipos más frecuentes de suelos naturalmente ricos en metales son: (i) suelos serpentiníticos que derivan de rocas ultramáficas ricas en Fe y Mg y enriquecidas en Ni, Cr y Co (ii) suelos calamínicos, ricos en Pb y Zn (iii) suelos enriquecidos en Cu y Co que derivan de argilitas y dolomitas y (iv) suelos derivados de rocas enriquecidas en Se.

Los suelos serpentiníticos se forman a partir de la meteorización y disolución de rocas ultramáficas ricas en minerales ferromagnesianos (70%) y con un contenido en sílice (SiO_2) menor del 45% (Kruckeberg, 2002). Estos suelos ocupan alrededor del 1% de toda la corteza terrestre (Proctor, 1999) y todos comparten algunas características específicas como son una relación Ca:Mg < 1, déficit de nutrientes esenciales (N, K o P) y elevadas cantidades de elementos traza como Ni, Cr o Co (Brooks, 1987). Estas propiedades junto con sus condiciones físicas dan lugar al denominado síndrome serpentinítico y proporcionan un ambiente inhóspito para la mayoría de las plantas, lo que ha dado lugar al desarrollo de adaptaciones fisiológicas y morfológicas diferentes a las de la flora de las áreas circundantes y a la presencia de altas tasas de endemismos (Whittaker, 1954). Entre la vegetación característica de estos suelos se encuentran las plantas

hiperacumuladoras, que se caracterizan por alcanzar concentraciones extremas de elementos traza en sus tejidos aéreos cuando crecen en sustratos ricos en metales (Brooks, 1987).

Actualmente, se conocen aproximadamente 500 taxones de plantas hiperacumuladoras de las cuales 450 acumulan Ni (Pollard *et al.*, 2014; van der Ent *et al.*, 2015). Una de las familias más ampliamente representada es la Brassicaceae que incluye el género *Alyssum*, en el cual se engloba aproximadamente el 90% de las especies hiperacumuladoras de Ni (Pollard *et al.*, 2014). En la Península Ibérica se encuentran dos subespecies endémicas de *Alyssum serpyllifolium* Desf. (Brassicaceae) hiperacumuladoras de Ni: *Alyssum serpyllifolium* ssp. *lusitanicum* (conocido como *A. pintodasilvae*), que crece de forma natural en el afloramiento serpentinítico de Barazón (NO España) y de Trás-os-Montes (NE Portugal) (Díez-Lázaro *et al.*, 2006; Freitas *et al.*, 2004; Pinto da Silva, 1970) y *Alyssum serpyllifolium* ssp. *malacitanum* (conocido como *A. malacitanum*) (López-González, 1975), que crece en las zonas serpentiníticas de Sierra Bermeja y de Sierra de Aguas (S España).

Contaminación de suelos por metales traza

Debido a que la formación del suelo es un proceso extremadamente lento y a que realiza funciones vitales se puede considerar como un recurso esencial y no renovable[1]. La Estrategia Temática para la Protección del Suelo de la Unión Europea incluye la contaminación del suelo entre los principales procesos de degradación. Su contaminación se define según la Comisión Europea como "la presencia de contaminantes en el suelo por encima de un cierto nivel que causa un deterioro o pérdida de una o más de sus funciones"[2]. Los datos recogidos por la Agencia Europea para el Medioambiente (EEA) estiman que el número de lugares potencialmente contaminados en la Unión Europea asciende a 2,5 millones y el de lugares contaminados a 342000[3]. En general, el sector de la producción contribuye más a la contaminación del suelo (60%) que el sector servicios (32%) y las actividades mineras junto con las industrias de metal son fuentes importantes de contaminación local (13%). En cuanto al tipo de contaminantes, los más frecuentes son aceites (24%) y metales pesados (35%) (Panagos *et al.*, 2013).

[1]http://ec.europa.eu/environment/soil/index_en.htm

[2]http://eusoils.jrc.ec.europa.eu/library/themes/contamination/

[3]EEA,http://www.eea.europa.eu/data-and-maps/indicators/progress-in-management-of-contaminated-sites-3/assessment

La mayoría de los países europeos poseen una legislación a nivel nacional o regional para regular la contaminación del suelo. Actualmente, en España, la contaminación del suelo está regulada por la Ley 5/2013 de Suelos Contaminados (BOE no. 140, 12 de Junio de 2013) y el Decreto Real 9/2005, ambos incluidos en el *II Plan Nacional de Suelos Contaminados* (2007-2015). Estos proporcionan una lista de actividades potencialmente contaminantes y los criterios para declarar un suelo como contaminado además de los Niveles Genéricos de Referencia (NGRs), que se definen como la concentración de un contaminante que no implica un riesgo para el ser humano y naturaleza. Los límites de metales incorporados al suelo en lodos de depuradora y compost destinados a la agricultura se regulan por el Real Decreto 1310/1990 (Ministerio de Agricultura 1990) y por la Ley española de fertilizantes (Real Decreto 824/2005 (Ministerio de Agricultura 2005)). Sin embargo, no existe todavía una legislación para el uso de enmiendas en suelos marginales o degradados.

Disponibilidad de metales y absorción por las plantas

La (bio)disponibilidad de los metal(oid)es se define como la fracción que puede interactuar con una diana biológica (Geebelen *et al.*, 2003). Las plantas obtienen los nutrientes y los metales traza desde la solución del suelo que está en contacto cercano con las raíces (definido por Hiltner en 1904 como rizosfera) y su disponibilidad está gobernada por un pseudo-equilibrio entre esta solución y la fase sólida (Kidd *et al.*, 2009). La cantidad de metal disponible está determinada, además de por la concentración total en la solución del suelo, por la capacidad de la fase sólida de reponer el elemento a la solución del suelo (Bruemmer *et al.*, 1986).

La reposición de los elementos incluye disociación de complejos presentes en la solución del suelo, pero también liberación de elementos lábiles asociados con la fase sólida, y está controlada por el tamaño de la fracción de metal lábil, su transporte y la velocidad a la que se libera desde la fase sólida (Lehto *et al.*, 2006). Las plantas modifican activamente las propiedades físicoquímicas de la rizosfera para movilizar nutrientes y/o metales traza. Los procesos que determinan la especiación de los metales en el suelo, su distribución y su disponibilidad están controlados por una combinación de las propiedades del suelo, las características de las plantas y la interacción con sus microorganismos asociados (Jones *et al.*, 2004).

La identificación de los mecanismos específicos que controlan la disponibilidad y absorción de metales en la rizosfera de las hiperacumuladoras puede ayudar a la mejora de los métodos de recuperación de suelos contaminados usando estas plantas. Aunque existen estudios que indican que la alta acumulación de metal por hiperacumuladoras es debida a que movilizan activamente los metales desde fracciones no disponibles (McGrath *et al.*, 1997; Kidd *et al.*, 2007; Alford *et al.*, 2010), la mayoría de los estudios demuestran que las plantas hiperacumuladoras acceden a las mismas fracciones que las no hiperacumuladoras (Salt *et al.*, 2000). Otros estudios más recientes sugieren que la liberación de ligandos orgánicos (como citrato) por las raíces de las hiperacumuladoras podría acelerar la disolución de los minerales ricos en Ni y mantener así una alta tasa de reposición desde las fracciones menos lábiles (Boominathan y Doran, 2003; Wenzel *et al.*, 2003; Kidd *et al.*, 2009). Finalmente, parece probable que las hiperacumuladoras no posean mecanismos específicos de movilización de metales sino que la disminución pronunciada de Ni que ocurre en su rizosfera como consecuencia de la alta tasa de acumulación por las plantas induzca su reposición desde fracciones menos móviles a otras más móviles.

Existe una gran variedad de métodos propuestos para evaluar la disponibilidad de los metal(oid)es en el suelo. Las técnicas tradicionales y más comúnmente utilizadas para estimar la fracción disponible de metales incluyen métodos químicos basados en extracciones simples y/o secuenciales (Michel y Ludwig, 2005; Hass y Fine, 2010). Sin embargo, la fracción lábil de metal estimada usando estas técnicas no se correlaciona con la acumulación por la planta. La técnica de DGT (del inglés *Diffusive Gradients in Thin Films*) ha sido desarrollada como una técnica pasiva para medir *in situ* el pseudo-equilibro que ocurre entre los metales en la disolución del suelo y la fracción intercambiable (Davison y Zhang, 1994), imitando el efecto de la planta. La técnica de la DGT se basa en la acción de una resina embebida en un gel que adsorbe continuamente iones desde la solución del suelo, lo que genera un gradiente entre el suelo y la resina. Mientras está en contacto con el suelo, la resina induce un flujo continuo de metales y la disociación desde la fase sólida para compensar la depleción en la solución en contacto con el gel. Se ha observado que la fracción de metal disponible estimada usando esta técnica se correlaciona mejor con la acumulación por parte de las plantas que la medida con extracciones químicas (Davison y Zhang, 2000; Degryse *et al.*, 2009).

Las medidas realizadas por DGT se pueden incorporar a modelos numéricos para estimar los parámetros que cuantifican la reposición de los metal(oid)es desde la fase sólida a la solución del suelo (Harper *et al.*, 1998; Ernstberger *et al.*, 2002). El modelo de DIFS (del inglés *DGT Induces Fluxes in Sediments and Soils*) se ha desarrollado para cuantificar los procesos que influencian el flujo de elementos hacia el gel de la DGT, es decir, para describir la interacción del gel con el suelo. Este modelo estima la proporción de elementos, en la fase sólida (K_D) que pueden liberarse y acumularse en el gel, y el tiempo de respuesta (T_C) al que se alcanza el equilibrio entre la fase sólida y la solución del suelo (Ernstberger *et al.*, 2002).

Tecnologías de recuperación de suelos respetuosas con el medio ambiente

En las últimas décadas se ha documentado un incremento en el uso de tecnologías verdes respetuosas con el medio ambiente (GRO, del inglés *Gentle remediation options*) para la recuperación de suelos contaminados. Estas opciones incluyen la estabilización del contaminante *in-situ* y las técnicas de fitocorrección que se basan en el uso de plantas y sus microrganismos asociados para la recuperación de suelos contaminados y se consideran más rentables y menos invasivas que las técnicas convencionales de ingeniería civil (Vangronsveld *et al.*, 2009; Mench *et al.*, 2010). Las tecnologías más comúnmente utilizadas en el caso de contaminación con metales pesados son: fitoestabilización, que incluye el uso de plantas exclusoras de metales en combinación con enmiendas del suelo para reducir la transferencia del contaminante a la cadena trófica; y fitoextracción, que implica el cultivo de plantas acumuladoras de metales que se cosechan al final de su ciclo de crecimiento retirando de esta forma el metal del suelo. Cuando el metal fitoextraído se puede recuperar y comercializar, obteniendo un beneficio económico, el proceso se denomina fitominería (Chaney *et al.*, 2007; Tang *et al.*, 2012). El éxito de las técnicas de fitocorrección es altamente dependiente de la solubilidad y la especiación de los metal(oid)es en el suelo y de la tolerancia de las plantas y su capacidad de acumular los elementos, por lo tanto, una adecuada selección de las plantas para cada caso es de vital importancia. En las técnicas de fitoextracción es deseable el uso de plantas con alta capacidad de acumulación de los elementos traza mientras que para la fitoestabilización se requieren plantas que excluyan los metales de su parte aérea.

Mejora de la eficiencia de técnicas de fitocorrección

Las plantas seleccionadas en técnicas de fitocorrección para la revegetación de suelos contaminados deben tolerar, además de la contaminación, otros numerosos factores de estrés tales como deficiencia de agua y nutrientes, acidez o salinidad, erosión o compactación, inundaciones, herbivoría o plagas. Las áreas contaminadas acogen una importante variedad de genotipos vegetales tolerantes a metales y adaptados a las condiciones físico químicas y climatológicas de cada sitio en particular (Batty, 2005). Las plantas hiperacumuladoras tienen una gran relevancia en el campo de la fitoextracción debido a su potencial aplicación en técnicas de fitominería. Actualmente, el Ni y el Co son los metales que presentan el precio más alto en el mercado; sin embargo, debido al alto número y amplia distribución de las hiperacumuladoras de Ni, este parece ser el elemento que puede ser más fácilmente explotado mediante fitominería (Chaney *et al.*, 2014). A pesar de que las hiperacumuladoras tiene características muy adecuadas para su empleo en fitoextracción, su aplicación todavía es limitada debido a su baja producción de biomasa o al desconocimiento sobre su cultivo (Kidd *et al.*, 2015). Ante esta limitación, se han descrito cultivos de alta biomasa como especies anuales de alto crecimiento (p.ej. girasol (*Helianthus annus*) o tabaco (*Nicotiana tabacum*)) o especies leñosas (p.ej. *Salix* spp. o *Populus* spp.), como una alternativa viable, particularmente para su uso en suelos contaminados con Cd, Zn o Se. Entre las ventajas de estas especies cabe mencionar que se pueden cultivar fácilmente utilizando practicas agronómicas ya establecidas (Ghosh y Singh, 2005; Meers *et al.*, 2005) y que su desarrollado sistema radicular permite la exploración de un mayor volumen de suelo y un crecimiento rápido. En cualquier caso, el cálculo del tiempo necesario para la recuperación del suelo es demasiado elevado y por lo tanto el proceso solo sería aceptable si se combina con producción de biomasa para bioenergía o como materia prima. En el caso de las técnicas de fitoestabilización, a menudo incluyen revegetación con plantas tolerantes y también incorporación de enmiendas inorgánicas y/u orgánicas para mejorar las propiedades físicas, químicas y biológicas del suelo (Vangronsveld *et al.*, 1996; Barrutia *et al.*, 2011). Las enmiendas que se utilizan de forma más habitual incluyen agentes de encalado, fosfatos, oxihidróxidos de Fe y Mn, materiales orgánicos, zeolitas, cenizas… (Adriano *et al.*, 2004; Bolan *et al.*, 2014). Estas enmiendas pueden reducir la solubilidad de los metal(oid)es por la formación de precipitados insolubles o aumentando la capacidad de adsorción del suelo. Además, en el caso de la materia orgánica, su uso puede mejorar las propiedades físicas del suelo como la

infiltración y retención de agua, proporcionar micro- y macro-nutrientes para el crecimiento vegetal y estimular la actividad microbiana.

Debido a que la deficiencia de nutrientes en los suelos contaminados es frecuentemente uno de los factores más limitantes para el desarrollo vegetal (Ye *et al.*, 2002), la fertilización de este tipo de suelos es un paso crucial para el establecimiento de una cobertura vegetal. Los regímenes de fertilización pueden estar diseñados para mejorar el crecimiento pero, en el caso de la fitoextracción, también para aumentar la acumulación de los elementos traza ya que la fertilización inorgánica puede alterar la disponibilidad de los elementos traza en el suelo (McLaughlin *et al.*, 1999; Singh *et al.*, 2011).

El diseño de patrones de cultivo apropiados puede mejorar significativamente el crecimiento de las plantas y su nutrición, además de modificar la solubilidad de los metales en el suelo (Kidd *et al.*, 2015). El cultivo intercalado de plantas se basa en interacciones interespecíficas entre raíces, que puede resultar en una mejora en la disponibilidad de los nutrientes y, por lo tanto, aumentar la productividad de los cultivos (Wieshammer *et al.*, 2007). Por otra parte, el intercalado de distintas especies puede alterar las condiciones en las rizosferas y afectar la disponibilidad de los metales a las plantas vecinas (Tang *et al.*, 2012). Además de por una razón práctica (evitar el agotamiento de nutrientes o pestes relacionadas con una especie determinada), el cultivo el intercalado con legumbres herbáceas (p.ej. *Lotus* spp.) o leñosas (e.g. *Alnus* spp.) puede mejorar la fertilidad del suelo ya que son fijadoras de N_2 y además algunas especies, como *Lupinus* spp., son también capaces de solubilizar P (Vance *et al.*, 2003; Liu *et al.*, 2013).

El uso de bacterias promotoras del crecimiento vegetal (PGPB, del inglés *plant growth promoting bacteria*) se ha descrito también como una opción para mejorar el establecimiento y crecimiento vegetal en suelos contaminados (Weyens *et al.*, 2009; Sessitsch *et al.*, 2013). Las bacterias asociadas a plantas incluyen bacterias endofíticas (que colonizan el interior de los tejidos vegetales sin causar efectos negativos en la planta), bacterias de la filosfera (que se encuentran en las superficies externas de las plantas) y bacterias rizosféricas (que habitan en el suelo en contacto con las raíces e influenciado por la planta). El uso de bacterias como biofertilizantes (p.ej. bacterias fijadoras de N_2 atmosférico, o capaces de solubilizar P o Fe) es una práctica común en agricultura para evitar el uso excesivo de fertilizantes químicos (Weyens *et al.*, 2009). Además, existen bacterias también capaces de producir fitohormonas que estimulan el crecimiento

y desarrollo vegetal (p.ej. ácido indolacético (AIA)) o que reducen el estrés vegetal mediante la reducción de niveles tóxicos de etileno mediante la síntesis de ACC-deaminasa (Glick, 2014). Las comunidades microbianas rizosféricas pueden afectar también a la disponibilidad de los metales en el suelo movilizándolos (acidificación, complejación o disolución) o inmovilizándolos (secuestro intracelular, precipitación o adsorción en la pared celular o exopolímeros). Diversos estudios han demostrado el uso positivo de inóculos bacterianos en técnicas de fitoextracción (usando hiperacumuladoras (Abou-Shanab *et al.*, 2006; Cabello-Conejo *et al.*, 2014) o especies acumuladoras de alta biomasa (Kuffner *et al.*, 2008; Becerra-Castro *et al.*, 2012)) o de fitoestabilización (Salt *et al.*, 1999; Dary *et al.*, 2010; Touceda-González *et al.*, 2015). De forma similar a lo que sucede con la selección de plantas, el uso de bacterias tolerantes y adaptadas a las condiciones del suelo es un pre-requisito para lograr una inoculación efectiva. Los ambientes ricos en metales son una fuente potencial de este tipo de microorganismos y su aislamiento y caracterización permite el descubrimiento de microrganismos adecuados para cada caso de estudio, además del establecimiento de colecciones de cepas bacterianas con potenciales aplicaciones en técnicas de fitocorrección.

OBJETIVOS Y PRINCIPALES TAREAS REALIZADAS

Los objetivos de esta Tesis se pueden resumir en los siguientes:

1. Estudio del sistema planta-suelo-bacteria en subespecies de Alyssum serpyllifolium *hiperacumuladoras de Ni*

a. Aislar y caracterizar (tanto fenotípica- como genotípicamente) bacterias cultivables asociadas con distintas poblaciones de las dos subespecies de hiperacumuladoras de Ni (*Alyssum serpyllifolium* subsp. *lusitanicum y* subsp. *malacitanum*) endémicas de áreas serpentiníticas de la Península Ibérica y comparar estos aislados con los obtenidos de la rizosfera de las no hiperacumuladoras *Dactylis glomerata* y *Santolina semidentata* (que crecen en las mismas zonas serpentiníticas) y *Alyssum serpyllifolium* ssp. s*erpyllifolium* (de un suelo calcáreo en el S de la Península Ibérica).

b. Evaluar la influencia de la hiperacumuladora *A. serpyllifolium* subsp. *lusitanicum* y la exclusora *Holcus lanatus* (procedentes de mismo suelo serpentinítico) en la disponibilidad de Ni en la rizosfera. Para estudiar la disponibilidad de Ni y su fraccionamiento se utilizaron extracciones convencionales mientras que las cinéticas de Ni y la capacidad del suelo de reposición de este

elemento se determinaron mediante la técnica de DGT y el modelo DIFS. La técnica de DGT se utilizó también para estudiar la distribución del Ni lábil a lo largo del eje radicular de *A. pintodasilvae* y *H. lanatus* creciendo en sistemas de rizotron.

2. Mejora de la eficiencia de las técnicas de fitoextracción usando enmiendas y/ o inoculación bacteriana

a. Evaluar el uso de residuos municipales compostados en la eficiencia de fitoextracción de distintas hiperacumuladoras de Ni. Se estudiaron los efectos de distintos regímenes de fertilización (compost o fertilización inorgánica) en la producción de biomasa y extracción de Ni de las hiperacumuladoras, *Alyssum serpyllifolium* subsp. *lusitanicum* (poblaciones de Samil (Portugal) y Barazón (NE, España)), *A. serpyllifolium* subsp. *malacitanum* (poblaciones de Sierra Bermeja y Sierra de Aguas (S España), *A. bertolonii* (población de Imprunetta, Italia) y *Noccaea goesingensis* (población de Redlschlag, Austria).

b. Evaluar el efecto de la combinación de enmiendas orgánicas e inóculos bacterianos en la mejora de la fitoextracción de cultivos de crecimiento rápido: *Salix caprea* y *Nicotiana tabacum*. En este contexto los objetivos específicos fueron (i) evaluar la capacidad de ambas especies para acumular metales traza al crecer en un suelo de mina contaminado con Cd, Pb y Zn; (ii) evaluar las mejoras en las propiedades del suelo, crecimiento vegetal, estado fisiológico y acumulación de metal después de la adición de compost y (iii) estudiar el uso de bacterias promotoras del crecimiento vegetal para mejorar la capacidad fitoextractora de las plantas.

c. Evaluar el uso de distintos métodos de inoculación bacteriana en plantas de *Nicotiana tabacum* establecidas en el suelo de mina descrito anteriormente. El principal objetivo fue estudiar si el modo de inoculación o la dosis de inóculo empleada pueden influenciar la respuesta de la planta a la inoculación. Para ello se evaluaron los efectos en la producción de biomasa y acumulación de metal de (i) inoculación en semilla o en suelo, (ii) inóculos con distintas densidades bacterianas, e (iii) inoculación única o repetida.

3. Monitorización de la eficacia a corto plazo de técnicas de fitoextracción y fitoestabilización en estudios pilotos en campo

a. Establecer una cobertura vegetal saludable (usando especies leñosas de *Salix* spp. o *Populus* spp. o la gramínea *Agrostis capillaris*) en escombreras de

minas altamente contaminadas con Cu mediante la reducción de la disponibilidad de metales y la mejora de la fertilidad del suelo. Para lograr este objetivo se estableció en la primavera de 2011 un ensayo de fitoestabilización en escombreras contaminadas con Cu en el NO de España. Las escombreras se enmendaron con residuos sólidos urbanos compostados y se plantaron con *Salix* spp., *Populus nigra* o *Agrostis capillaris.*

b. Estudiar el uso de especies leñosas de alta biomasa, *Salix smithiana* o *S. atrocinerea*, y de una hiperacumuladora de Cd/Zn, *Noccaea caerulescens*, en técnicas de fitoextracción en un suelo de mina contaminado con Pb/Zn en el NO de la Península Ibérica. Se evaluó también el efecto de la adición de enmiendas orgánicas (compost), fertilización inorgánica y distintos patrones de cultivo (monocultivo o cultivo intercalado con las leguminosas *Alnus glutinosa* y *Lotus corniculatu*s) en las propiedades físico- y bio-químicas, crecimiento vegetal, estado nutricional y acumulación de metal.

PRINCIPALES RESULTADOS Y CONCLUSIONES

1.a. Las densidades bacterianas (Unidades Formadoras de Colonias (UFC) g^{-1} suelo) en los suelos serpentiníticos no se diferenciaron entre las distintas zonas de estudio (Barazón (L), Morais (M), Samil (S) y S. Bermeja (SB) y variaron desde 3.7 x 10^7 UFCs g^{-1} suelo en L hasta 7.9 x 10^7 UFCs g^{-1} suelo en SB. Por otra parte, las densidades en los suelos calcáreos de S. Nevada (SN) fueron hasta 10 veces mayores (5.2 x 10^8 UFCs g^{-1} suelo) que en los suelos serpentiníticos. Las densidades bacterianas en las rizosferas de todas las especies (especialmente en *Alyssum* ssp.) fueron mayores que las observadas en el suelo no vegetado, con la excepción de *D. glomerata* en M.

En los suelos serpentiníticos los recuentos de bacterias resistentes a Ni fueron como máximo un orden de magnitud menor que los recuentos bacterianos totales mientras que, en el suelo calcáreo, la densidad de bacterias resistentes a Ni fue hasta 3 órdenes de magnitud menor que la de bacterias totales. Por otra parte se encontró una mayor densidad de bacterias resistentes a Ni en la rizosfera de las plantas hiperacumuladoras que en el suelo no vegetado o en la rizosfera de no hiperacumuladoras Se obtuvieron un total de 550 aislados bacterianos con distintos morfotipos de colonia, en los cuales las características fenotípicas más frecuentes fueron la producción de ácidos orgánicos y ácido indolacético (AIA) (29.3% y 23.1%, respectivamente), características que se detectaron en cepas asociados a todas las especies vegetales. Las cepas bacterianas productoras de

sideroforos fueron especialmente abundantes en la rizosfera de *D. glomerata* (51.2% en M), mientras que la capacidad de solubilizar fosfatos o producir biosurfactantes fueron características relativamente raras entre los aislados (4.5% y 0.5% respectivamente). El índice de diversidad Shannon (H´), calculado en base a los patrones de BOX-PCR, mostró que la diversidad de las cepas bacterianas con alto nivel de tolerancia a Ni fue mayor en la rizosfera de la hiperacumuladora de Ni que en las no-acumuladoras. Los 329 aislados secuenciados se distribuyeron entre cuatro filos diferentes, dos que corresponden a bacteria Gram-positivas (Actinobacteria y Firmicutes), y dos a Gram-negativas (Bacteroidetes y Proteobacteria). La abundancia de cepas de estos filos varió dependiendo del tipo de suelo, el 94% de los aislados de los suelos serpentiníticos fueron bacterias Gram-positivas, mientras que en el suelo calcáreo las bacterias Gram-negativas representaron el 23% de la colección. Algunos géneros como *Arthrobacter*, *Streptomyces* y *Rhodococcus* se encontraron en la rizosfera de todas las especies, mientras que otros géneros como *Stenotrophomonas*, *Methylobacterium*, *Staphylococcus*, *Amycolatopsis* y *Mycobacterium*, además de representantes del filo *Bacteroidetes* se encontraron solo asociados a las hiperacumuladoras.

1.b. En comparación con el suelo no vegetado, tanto la hiperacumuladora *A. pintodasilvae* como la exlusora *H. lanatus* aumentaron la cantidad de carbono orgánico disuelto, la capacidad de intercambio catiónico y aumentaron la relación Ca/Mg del suelo rizosférico . Asimismo, la disponibilidad de Ni (estimada usando extracciones simples ($Sr(NO_3)_2$ y $Ca(NO_3)_2$ y la técnica de DGT) fue mayor en la rizosfera que en el suelo no vegetado y se observó un cambio en el fraccionamiento de Ni (ambas especies aumentaron la fracción fácilmente disponible y además la hiperacumuladora aumentó significativamente la fracción de Ni unida a materia orgánica y a óxidos de Fe amorfos). Todos estos cambios fueron más pronunciados en la rizosfera de la hiperacumuladora, y la técnica de la DGT fue más sensible a los cambios inducidos por las plantas en la disponibilidad de los elementos que las extracciones convencionales. En cuanto a la disponibilidad de otros cationes importantes en este suelo, en general, la rizosfera de la hiperacumuladora mostro un enriquecimiento en elementos como Ca, K, Co, Mn o Al. Tanto en el caso del suelo no vegetado como en el rizosférico, la a cantidad de Ni acumulada en el gel de DGT aumentó al aumentar el tiempo de contacto entre el suelo y el gel y este aumento fue más pronunciado en el caso de la hiperacumuladora.

La modelización de los datos de DGT mostró que en la rizosfera de *H. lanatus* es posible sostener la baja concentración inicial de Ni soluble presente en

este suelo mediante la reposición desde la fase sólida, mientras que la reposición de Ni en la rizosfera de la hiperacumuladora no fue suficiente para mantener la alta concentración inicial de Ni soluble en el tiempo. En presencia de la planta hiperacumuladora, que claramente ejerció un efecto más pronunciado en la disponibilidad de Ni en el suelo, es posible que se pudiera sostener la concentración de Ni medida mediante DGT en el tiempo. El uso de los geles de DGT para el estudio de la distribución de Ni a lo largo de la raíz de *A. pintodasilvae* y *H. lanatus* mostró una zona de disminución de Ni alrededor de la raíz de ambas especies; aunque la intensidad de esta disminución fue ligeramente mayor alrededor de la raíz de la hiperacumuladora. Los resultados de este estudio mostraron una correlación importante entre el Ni disponible en el suelo y el carbono orgánico disuelto, y también se observó una co-solubilizacion de Ni junto con otros nutrientes que podría estar relacionada con la nutrición de la hiperacumuladora.

2.a. Tanto la fertilización inorgánica (NPK) como la adicción de enmiendas orgánicas (tres cantidades de compost: 2,5; 5 y 10% p/p) a suelos serpentiníticos mejoraron de forma pronunciada la cantidad total de Ni fitoextraído por las cinco hiperacumuladoras (*Alyssum pintodasilvae*, *A. malacitanum*, *A. bertolonii* y *Noccaea goesingensis*) estudiadas. El principal cambio inducido por la fertilización inorgánica en el suelo serpentinítico fue un aumento en el P disponible, mientras que la adición de compost aumentó la cantidad de C, N, P, la capacidad de intercambio catiónico y la ratio Ca/Mg y, al mismo tiempo, causó una reducción importante en la disponibilidad de Ni en el suelo. Estos efectos fueron en general más pronunciados con un aumento en la tasa de compost añadido.

En el suelo sin tratar no existieron diferencias significativas entre las distintas (sub)especies de hiperacumuladoras en la producción de biomasa (entre 11,6 mg planta^{-1} (*A. pintodasilvae* de Samil) y 33,8 mg planta^{-1} (*N. goesingensis*)). En cuanto a la acumulación de Ni, la concentración más alta correspondió a la población de *A. malacitanum* de S. Bermeja (9523 mg kg^{-1}). Todos los tratamientos (fertilización inorgánica y compost) aumentaron el crecimiento y biomasa vegetal y este efecto fue especialmente pronunciado en *N. goesingensis* (la adición de 5% de compost aumentó hasta 162 veces su biomasa). Generalmente se observaron los mayores incrementos con las dosis de 2,5 o 5% de compost tanto en *Alyssum* como en *Noccaea*. En las (sub)especies de *Alyssum*, el mayor efecto se observó en *A. bertolonii*. Al contrario de lo que se vio con la producción de biomasa, pero de acuerdo con la disponibilidad de Ni en el suelo, las enmiendas de compost

redujeron significativamente las concentraciones de Ni en la parte aérea de todas las plantas. Por otra parte, la fertilización inorgánica aumentó ligeramente la acumulación de Ni en la población de Samil de *A. pintodasilvae*. En general, todos los tratamientos mejoraron también el estado nutricional de las plantas, aumentando la acumulación de P, K y Ca (únicamente en el caso de la enmienda de compost). La cantidad total de Ni fitoextraído desde el suelo aumentó significativamente tanto con la enmienda orgánica como con la fertilización inorgánica, pero los resultados más prometedores se encontraron usando cantidades intermedias de compost (2,5 y 5%).

2.b. La adición de compost (5% p/p) a suelos de mina contaminados con Zn, Cd y Pb aumentó su contenido en nutrientes (C, N o P) a la vez que causó una reducción en la capacidad de intercambio catiónico (debido principalmente a una reducción del Ca intercambiable) y en la concentración de metales disponibles en el suelo. Después de tres meses creciendo en el suelo de mina sin enmienda, la biomasa de *S. caprea* fue hasta cinco veces mayor que la de *N. tabacum*. En cuanto a la acumulación de metales, la concentración de Zn en hojas de *S. caprea* (2050 mg kg^{-1}) fue casi cinco veces mayor que la concentración en tallo, y también significativamente mayor que la concentración en la parte aérea de tabaco (725 mg kg^{-1}). Por el contrario, la concentración de Cd en la parte aérea de tabaco (media de 12,2 mg kg^{-1}) fue mayor que en las hojas o tallo de *S. caprea*. En cuanto al Pb, *S. caprea* prácticamente no acumuló Pb en sus tejidos aéreos mientras que las plantas de tabaco acumularon una media de 6,7 mg kg^{-1}. Ambas especies vegetales crecieron mejor en el suelo enmendado con compost pero este efecto fue especialmente pronunciado en tabaco (hasta 85% de aumento). Al mismo tiempo, la adición de compost causó una disminución en las concentraciones de Zn, Cd y Pb en la parte aérea de tabaco y en el tallo de *S. caprea*. Por el contrario, el compost aumentó la acumulación de Zn y Cd en las hojas de S. *caprea*. A consecuencia de la promoción en el crecimiento inducida por el compost en plantas de *N. tabacum* se llegó a triplicar la cantidad total de Zn, Cd y Pb extraído. Al comparar las dos especies estudiadas se vio que, en el suelo sin compost, las plantas de *Salix* fueron capaces de extraer hasta tres veces más Cd y Zn que las de tabaco. En el caso de las plantas de *Salix* creciendo en suelo enmendado con compost, la cantidad total de Cd disminuyó con respecto a las plantas en suelo sin enmienda, a la vez aumentó el Zn fitoextraído (que fue debido a una mayor acumulación y no a un mayor crecimiento de las plantas).

La inoculación bacteriana tuvo un efecto significativo sobre el crecimiento

de tabaco, sobre todo en suelos enmedados con compost en los que todos los inóculos (P64, P87, P30, P75 y P29) aumentaron significativamente su biomasa. En el caso de *S. caprea* la inoculación bacteriana tuvo un menor efecto y solo la cepa P30 causó un ligero aumento en su crecimiento. La inoculación bacteriana también mejoró algunos índices de eficiencia fotosintética como CHL o Fv / Fm en plantas de tabaco creciendo en suelo sin compost. Los inóculos bacterianos también influenciaron la disponibilidad de metales en el suelo y este efecto fue más pronunciado en los suelos sin enmienda orgánica. Todas las cepas en combinación con plantas de tabaco aumentaron el Zn y Cd disponible en estos suelos. En el caso de los suelos plantados con *Salix*, la variación en la disponibilidad de metales dependió del inóculo y de la presencia de compost. Las cepas bacterianas inoculadas también modificaron el fraccionamiento de metales en ambos suelos. En cuanto a la acumulación de metal en planta, en suelos sin enmienda, las cepas P30, P75 y P29 disminuyeron la concentración de metales en las plantas de tabaco y las cepas P64 y P87 aumentaron la concentración de Zn. En las plantas creciendo en suelo con compost la inoculación bacteriana no causó efectos significativos en la concentración de Zn mientras que las cepas P30 y P29 disminuyeron la concentración de Cd. En el caso de Pb, en general las cepas tendieron a aumentar su acumulación. En las plantas de *S. caprea*, se los efectos inducidos por las bacterias fueron más pronunciados en las hojas. Así por ejemplo en plantas desarrolladas en suelo sin enmienda, la cepa P87 aumentó la acumulación de Cd y Zn en hojas.

Los efectos de las bacterias en la cantidad total de metal fitoextraído dependieron del tipo de suelo en el que crecieron las plantas. En el caso de las plantas de tabaco, en de los suelos sin enmendar, la cantidad de metal extraído se vio negativamente afectada por la inoculación bacteriana, pero en las plantas creciendo en suelos enmendados con compost, todos los inóculos mejoraron la capacidad de extracción de metales. Por el contrario, en el caso de *S. caprea*, los efectos beneficiosos de la inoculación bacteriana fueron más pronunciados en el suelo sin tratar, mientras que en el suelo con compost, en general, los inóculos redujeron la cantidad de Zn y Cd fitoextraído.

2.c. El efecto de la inoculación de la cepa P30 de *Rhodococcus erythropolis* en el crecimiento de *N. tabacum* dependió tanto de la densidad celular del inóculo como del modo de inoculación (en semilla, en suelo, aplicada una o dos veces, o aplicada en semilla y en suelo). El tratamiento que produjo un mayor aumento en la biomasa de las plantas de tabaco fue una única inoculación en suelo con la

densidad de 10^6 UFCs mL^{-1} (hasta 45% de aumento). La inoculación repetida en suelo usando la densidad de 10^6 UFCs mL^{-1} también aumentó la producción de biomasa pero solo hasta un 9%. En cuanto a la acumulación de metales por las plantas de tabaco, los cambios detectados dependieron más del modo de inoculación que de la densidad del inóculo. El aumento de densidad desde 10^6 UFCs mL^{-1} a 10^8 UFCs mL^{-1} no dio lugar a una mejora en los efectos. La inoculación bacteriana afectó en menor medida a las concentraciones de metales en la parte aérea que a la producción de biomasa, aunque se observó un efecto positivo en la acumulación de Pb con el inóculo 10^8 UFCs mL^{-1} aplicado en suelo (29% de aumento) o en semilla y suelo (34% de aumento). La acumulación de Zn también aumentó hasta un 24% después de la inoculación en semilla con una densidad de 10^6 UFCs mL^{-1}, mientras que en el caso de la acumulación de Cd, ningún tratamiento indujo un aumento significativo. Los tratamientos de inoculación también afectaron a la disponibilidad de metal en el suelo; sin embargo, los aumentos en la disponibilidad inducidos por las bacterias no siempre se vieron reflejados en un aumento de acumulación de metales por las plantas.

En general, la inoculación con la cepa P30 tuvo un efecto más beneficioso en el crecimiento de la planta que en la acumulación de metal. Todos los tratamientos aumentaron la cantidad total de metal fitoextraído por las plantas; sin embargo, los mayores incrementos en la cantidad total de metal extraído no se correlacionaron con un aumento en el número de bacterias aplicadas. De hecho, los mejores resultados se obtuvieron después de una inoculación única en el suelo utilizando la densidad celular más baja (10^6 UFCs mL^{-1}).

3.a. Los suelos de las escombreras de la mina de Touro presentaron un pH ácido y baja fertilidad (reflejada en un bajo contenido total de C y N o P disponible). La capacidad de intercambio catiónico (CIC) fue también baja y dominada por H^+ y Al^{3+}. Las concentraciones de elementos traza fueron muy variables en las escombreras pero, en general, se encontraron elevadas concentraciones de Al, Cr, Fe, Mn o Ni y los valores más elevados de metales disponibles (extraídos con $NaNO_3$) correspondieron al Cu (media de 61 mg kg^{-1} y rango entre 7 y 219 mg kg^{-1}) y Mn (media de 823 mg kg y rango desde 650 a 1142 mg kg^{-1}). Un año después de la adición de compost, el pH del suelo aumentó significativamente y este aumento continuó observándose durante los dos siguientes años, y también se observó un aumento progresivo en C y N con el tiempo, encontrándose los máximos valores a los dos años de añadir la enmienda de compost. Asimismo, el compost causó cambios significativos de la CIC, que aumentó significativamente

un año después del tratamiento y descendió de nuevo después de tres años a valores similares a los que se determinaron en los suelos sin enmienda. A pesar de esto, mientras que antes de la adición del compost, la CIC estaba dominada por H^+ y Al^{3+} (con una saturación en base menor al 20%), tres años después del tratamiento la saturación en bases fue superior al 90% (dominada principalmente por Ca^{2+}). El P disponible también aumentó significativamente después de la adición del compost (hasta 100 veces un año después de la aplicación de la enmienda). Al mismo tiempo, la adición de compost disminuyó significativamente la disponibilidad de elementos traza como Cu, Al, Cd, Co, Cr, Ni, Zn o Mn. La actividad enzimática fue prácticamente nula en el suelo sin enmendar; sin embargo, un año después de la adición de compost, la actividad de las enzimas hidrolasas aumento hasta 23 veces y los aumentos fueron incluso más importantes en ureasa y arilsulfatasa. En general, las actividades enzimáticas altas se mantuvieron durante los tres años de experimento. El crecimiento vegetal mostró una alta variabilidad entre los individuos de la misma especie a lo largo de la parcela experimental. El máximo crecimiento en la primera temporada de crecimiento se observó en las plantas de *S. viminalis* (106 cm), que mostraron también la mayor altura en el momento de la cosecha después de cuatro años de crecimiento (media de 219 cm con plantas que alcanzaron hasta 390 cm). Después de cuatro años el porcentaje de mortalidad fue mayor y más variable en *Salix* spp. que en *P. nigra*, por consiguiente, la biomasa final obtenida fue muy variable entre sub-parcelas. El valor medio de biomasa obtenida para *Salix* spp. fue de 7,0 ±3.0 t ha^{-1} en *S. caprea* y 4.3 ±2.3 t ha^{-1} en *S. viminalis*. La producción de biomasa de *P. nigra* fue más uniforme con una media de 4.3 ±0.7 t ha^{-1}. La gramínea *A. capillaris* presentó un buen establecimiento y cobertura después de su siembra y la producción aérea de biomasa fue de 2.2 t ha^{-1} en la primera cosecha (dos años de crecimiento) y aumentó hasta 4.6 t ha^{-1} en la segunda cosecha (tres años de crecimiento). La concentración de nutrientes en los tejidos aéreos se mantuvo dentro del rango considerado beneficioso para el crecimiento vegetal y la concentración de Cu no excedió el umbral considerado como fitotóxico. Los principales efectos en los parámetros fisicoquímicos del suelo fueron un resultado directo de la adición de compost, mientras que los efectos inducidos por el crecimiento de las plantas se observaron en los parámetros bioquímicos. A pesar de esto, en comparación con los no plantados, en los suelos plantado también se observaron mejoras en parámetros como el pH, la CIC o la disponibilidad de Cu .

3.b. Los suelos de la mina de Rubiais presentaron un pH neutro o

ligeramente alcalino (7.1-8.6) y baja fertilidad, reflejada en una baja cantidad de C, N y P disponible. Las concentraciones de metales en la zona de estudio fueron altamente variables a lo largo de las parcelas pero, en general, se encontraron altas concentraciones de Cd, Pb y Zn. La adición de 5% (p/p) de compost aumentó significativamente la cantidad de nutrientes, a la vez que disminuyó la cantidad de metales disponibles en el suelo (debido a un cambio en la distribución de los metales en el suelo hacia fracciones menos disponibles asociadas a óxidos de Fe y Mn, materia orgánica y la fracción residual). La adición de compost mejoró también las propiedades bioquímicas del suelo (como demuestra el aumento en la actividad enzimática).

En los suelos sin enmienda, el crecimiento de ambas especies de *Salix* spp., provocó un aumento de C y N en el suelo comparado con los suelos sin plantar. En el caso de plantas desarrolladas en los suelos fertilizados con NPK se observó una disminución significativa en el pH del suelo, al mismo tiempo que un aumento en la biodisponibilidad de Cd y Zn. En el caso de plantas de *S. smithiana* en co-cultivo con *A. glutinosa*, en los suelos enmendados se observó también un aumento en la disponibilidad de metales. En general, las actividades enzimáticas fueron mayores en los suelos con planta y no dependieron de la especie de *Salix* utilizada; sin embargo, en comparación con el monocultivo, el co-cultivo de *S. smithiana* con *A. glutinosa*, en general, aumentó la actividad enzimática. En el caso de la hiperacumuladora *N. caerulescens*, su crecimiento no modificó la cantidad de C, N o P en los suelos sin enmienda, pero aumentó significativamente estos nutrientes en los suelos con compost (comparado con el suelo sin plantar) y disminuyó la cantidad de Zn disponible tanto en los suelos sin enmienda como en los enmendados. De forma similar a lo observado en el caso de las especies leñosas, *N. caerulescens* aumentó la actividad enzimática del suelo y, en algunos casos, este aumento fue más pronunciado en el co-cultivo con *L. corniculatus* que con la hiperacumuladora en monocultivo.

Los ejemplares de *S. smithiana* mostraron un buen establecimiento y baja mortalidad (4-28%), mientras que en el caso de *S. atrocinerea*, se encontró una menor supervivencia y las plantas fueron solo capaces de establecerse en el suelo sin enmienda y en monocultivo (en este tratamiento se observó un 84% de supervivencia). El crecimiento de individuos de una misma especie fue altamente variable a lo largo de la parcela experimental. Los individuos de *S. smithiana* (media de 82 cm después de cuatro años de crecimiento) fueron siempre mayores que *S. atrocinerea* (media de 42 cm después de cuatro años de crecimiento) y se

observó un efecto positivo de la fertilización al cabo de tres años en el crecimiento de las plantas. El co-cultivo con *A. glutinosa* provocó también un aumento en la altura de las plantas de *S. smithiana*. En cuanto a la acumulación de metales por las especies de *Salix*, las máximas concentraciones de Cd y Zn se observaron en hojas de *S. smithiana* desarrolladas en suelo sin enmendar (21.4 mg kg^{-1} y 4248 mg kg^{-1} respectivamente), y la adición de compost causó una reducción en la cantidad de metales acumulados por las plantas (la máxima concentración en hojas de *S. smithiana* fue 11.3 y 1782 mg kg^{-1} de Cd y Zn respectivamente).

N. caerulescens mostró un buen establecimiento en ambos tratamientos (con y sin enmienda) y una baja mortalidad (4-24%). El compost tuvo un efecto importante en el crecimiento de las hiperacumuladoras, aumentando significativamente su cobertura y producción de biomasa (desde 72 g m^{-2} hasta 210 g m^{-2} en monocultivo), mientras que el co-cultivo con la leguminosa disminuyó la cantidad de biomasa producida en los suelos sin enmendar. En los suelos sin compost, la máxima concentración de Cd y Zn se encontró en las hojas de *N. caerulescens* durante el periodo de floración (hasta 1350 mg kg^{-1} de Cd y hasta 22052 mg kg^{-1} de Zn). Del mismo modo que en el caso de las especies leñosas, la adición de compost disminuyó la acumulación de metales por las plantas; sin embargo, el cocultivo con *L. corniculatus* mejoró tanto el estado nutricional de las plantas como la acumulación de metales. La cantidad total de Zn extraído fue mayor en los suelos con compost (desde 586 mg m^{-2} hasta 998 mg m^{-2}), mientras que la enmienda no causó un efecto en la cantidad de Cd fitoextraído.

Las principales conclusiones de esta tesis son:

La selección de microorganismos adaptados a las condiciones del suelo y capaces de colonizar la rizosfera son pre-requisitos importantes para desarrollar técnicas de inoculación exitosas. El estudio de la rizosfera de las hiperacumuladoras permitió el aislamiento de bacterias con potencial en técnicas de re-inoculación. Un pequeño número de aislados se encontraron exclusivamente asociados a las subspecies hiperacumuladoras de *Alyssum* y podrían ser candidatos interesantes para estudios adicionales relacionados con la aplicación de especies hiperacumuladoras de Ni en fitominería.

El estudio de los mecanismos que controlan la disponibilidad de Ni en la rizosfera de las hiperacumuladoras es de vital importancia para el desarrollo de técnicas agronómicas enfocadas a la mejora de la fitoextracción (o fitominería)

mediante el incremento de la disponibilidad de Ni. La hiperacumuladora *A. pintodasilvae* mostró una mayor capacidad de influenciar las características físicoquímicas del suelo pero, contrariamente a lo esperado, en su rizosfera se apreció una menor capacidad de reposición de metal que en el caso de la excluso-ra *H. lanatus*. Los resultados de este estudio sugieren que la mayor disponibilidad de Ni en la rizosfera de la hiperacumuladora está relacionada con una mayor disolución mineral debido a la búsqueda activa de nutrientes por la planta. El carbono orgánico disuelto podría ser un parámetro determinante de la disponibilidad de metal en el suelo por lo que se necesitan más estudios para caracterizar su papel en la disponibilidad de Ni.

Los estudios realizados a escala de maceta en invernadero demuestran que el uso de técnicas agronómicas como la fertilización inorgánica o el uso de enmiendas orgánicas pueden mejorar las condiciones fisicoquímicas del suelo para el desarrollo vegetal en técnicas de fitoextracción. Además, el uso de subproductos de la industria como enmiendas en el suelo proporciona una forma económica de reciclaje de estos residuos.

Los estudios de bioaumento, también realizados a escala de maceta en invernadero, demostraron que en algunos casos los inóculos bacterianos pueden mejorar el crecimiento vegetal, modificar la disponibilidad del metal en el suelo y aumentar la acumulación de metal por las plantas. Sin embargo, estos efectos fueron altamente específicos dependiendo del tipo de suelo y/o especie vegetal. Además, en alguna de estas asociaciones planta-bacteria, se observaron efectos sinérgicos en el crecimiento vegetal cuando se combinaron enmiendas de suelo (compost) con la inoculación bacteriana. El método de inoculación mostró gran influencia en la respuesta de la planta a la inoculación. Los experimentos realizados permiten concluir que, tanto los modos de inoculación (semilla como en suelo) como la densidad celular del inóculo, son factores importantes a tener en cuenta a la hora de diseñar estrategias de inoculación bacteriana, y que es necesario lograr una optimización de estas estrategias antes de su aplicación a gran escala

El estudio de campo realizado en una antigua mina de Cu (Touro) demostró que la fisoestabilización asistida puede ser una técnica adecuada para disminuir los riesgos medioambientales asociados a escombreras de minas altamente contaminadas. En este estudio, el uso de compost de baja calidad (basados en residuos orgánicos y/o industriales) permitió el establecimiento y crecimiento en suelos degradados, tanto de especies de alta biomasa como de gramínea. Los

resultados del ensayo mostraron también que enmiendas orgánicas en combinación con crecimiento de especies exclusoras pueden ser útiles en la reducción de la disponibilidad de Cu , la mejora la fertilidad y de la actividad biológica del suelo.

El estudio de campo llevado a cabo en una antigua mina de Pb/Zn (Rubiais) en el que se evaluó la eficacia de fitoextracción de *Salix* spp. y de la hiperacumuladora *N. caerulescens*, demostró que, tanto el régimen de fertilización (NPK o enmiendas orgánicas), como el patrón de cultivo (monocultivo o intercalado con legumbres), pueden modificar la fertilidad del suelo, la actividad microbiana, el crecimiento vegetal, el estado nutricional y la acumulación de metales. El cultivo de la hiperacumuladora logró reducir la concentración de Zn disponible en el suelo después de solo un ciclo de crecimiento, y este efecto fue mayor en los suelos enmendados con compost en donde su crecimiento fue también mayor. Sin embargo, en el caso del cultivo de *Salix* spp. se necesitaría un mayor periodo de tiempo para observar los efectos de los tratamientos ensayados. Estos resultados refuerzan la necesidad de ensayos de campo de medio a largo plazo para la selección de las técnicas más adecuadas para cada lugar antes de su desarrollo a gran escala.

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1

INTRODUCTION



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1.1. Trace elements in soils

Trace elements (TE) refer to elements that occur in the environment at levels below 0.1% (1000 mg kg^{-1}) in natural media or below 0.01% (100 mg kg^{-1}) in plant or animal tissues (Adriano, 2001). Often synonymous for TEs are the terms trace metals or heavy metals. The term heavy metal defines elements with densities of > 5 g cm^{-3} while the term trace element is related to their abundance and includes elements of various chemical properties: metals and metalloids (Kabata-Pendias, 2010). Metal(loid)s are defined as substances with high electrical conductivity, luster, and malleability, which readily loses electrons to form positive ions (cations).

Several trace metals are essential micronutrients to plants and/or animals (such as copper (Cu), iron (Fe), manganese (Mn), nickel (Ni) and zinc (Zn)) and play a vital role in cellular processes and metabolism. For example, metal(loid)s are components of metalloproteins and act as cofactors of various enzymes implicated in essential metabolic processes, such as DNA or RNA synthesis. However, other elements such as arsenic (As), cadmium (Cd), mercury (Hg) and lead (Pb) are considered non-essential and lack any known biological function. While deficiencies in micronutrients can seriously disturb normal development of organisms, an excess of metal(loid)s can, in general, adversely affect biochemical reactions and physiological processes, causing a major risk for the environment and human health (Cuypers *et al.*, 2010; Nawrot *et al.*, 2010). For example, a decrease in biomass production has commonly been observed in plants subjected to elevated metal(loid) concentrations (Hirt, 2009). Interferences in photosynthesis, mineral nutrition and water balance have also been documented (Bernal *et al.*, 2007; Cuypers *et al.*, 2010; Emamverdian *et al.*, 2015). Phytotoxicity results from (i) the displacement of essential metal(loid) ions from biomolecules, (ii) blocking of essential functional groups of biomolecules and modification of its active conformation (iii) disruption of the integrity of biomolecules, (iv) modification of other biologically active agents, and (v) stimulation of the formation of free radicals and reactive oxygen species (ROS) (Hall, 2002). Plants grown on contaminated soils may have reduced productivity and plant TE uptake may facilitate the entry of toxic elements into the food chain, possibly affecting human nutrition. In humans, examples of extreme trace metal(loid) poisoning include the *Itai-itai* (Cd poisoning) and *Minamata* (Hg poisoning) diseases in Japan, or lead poisoning due to the use of leaded gasoline.

Natural concentrations or background levels of TE in soils are strongly dependent on the soil parent material and acting weathering processes (Adriano, 2001; Ross, 1994). An increasingly industrialised society has, however, led to the widespread introduction of trace metals and metalloids into our environment, causing acute and diffuse contamination of soil and waters.

Natural metal(loid)-enriched soils

The most important types of natural metalliferous soils include (i) serpentine soils, which are derived from Fe- and magnesium (Mg)-rich ultramafic rocks and are significantly enriched in metals such as, Ni, chromium (Cr) and cobalt (Co) (ii) calamine soils, which are derived from non-sulphide Zn ores in carbonate rocks and are enriched in Pb, Zn and usually contain elevated concentrations of Cd, (iii) Cu- and Co-containing soils, which are derived from argillites and dolomites, and (iv) soils derived from various selenium (Se)-rich rock types, such as black shales (Reeves, 2006; Winkel *et al.*, 2015).

Serpentine soils are formed through the weathering of ultramafic rocks which are comprised of at least 70% ferromagnesian (or mafic) minerals (particularly within the olivine and pyroxene groups) and less than 45% of silica (SiO_2) (Kruckeberg, 2002). These soils are ubiquitous, but patchily distributed around the world (occupying approximately 1% of the earth's crust (Proctor, 1999) and although some variations can occur between sites, serpentine soils are typically characterised by a low calcium (Ca):Mg ratio and elevated concentrations of TE such as Ni, Cr and Co (Table 1.1). Serpentine soils are also often deficient in essential nutrients such as nitrogen (N), potassium (K) or phosphorus (P) (Gordon and Lipman, 1926; Vlamis and Jenny, 1948; Walker, 1954; Proctor and Woodell, 1975; Brooks, 1987). Since many serpentine outcrops are steep and rocky their associated soils are often skeletal, with a low organic matter (OM) content and water holding capacity (WHC) (Brooks, 1987; Macías and Calvo de Anta, 2001; Roberts and Proctor, 2012). As a whole, the particular physical, chemical and biological properties of these soils are commonly referred to as the *serpentine syndrome* and present an inhospitable environment for most plant species. As a result, serpentine soils often support plant communities with high rates of endemism and which have evolved both morphological and physiological adaptations differentiating them from the flora of neighbouring areas (Whittaker, 1954; Proctor, 1971; Jenny, 1981). Within these plant communities a rare group of plants

can be found: the so-called metal-hyperaccumulators which will be discussed in the following section.

Table 1.1. Mean element concentrations (μg g^{-1}) in rocks, soils and vegetation

	Co	Cr	Cu	Pb	Mn	Ni	Zn
Earth´s crust	25	100	55	13	950	75	70
Granite	3	20	13	48	165	1	45
Basalt	47	114	110	8	1280	76	86
Ultramafic rocks	150	1600	10	1	1620	2000	50
Soil (non-ultramafic)	10	60	20	10	850	40	50
Soils (ultramafic)	250	2500	20	10	1000	2500	40
Vegetation (non-ultramafic)	1	1	10	10	80	2	100
Vegetation (ultramafic) *	10	10	10	10	100	80	100

*excluding hyperaccumulators
Source: Brooks, (1987) and Baker and Brooks, (1989)

Metal-hyperaccumulating plants

Metalliferous plants can be classified into three groups according to their response (in terms of uptake and accumulation) to elevated TE concentrations in the soil: excluders, indicators and (hyper)accumulators (Baker, 1981; Baker and Brooks, 1989) (Figure 1.1). Excluder plants are able to restrict the uptake of metal (loid)s into their roots and/or their translocation to the shoot, thereby maintaining a low level of metal(loid)s in their aerial tissues over a wide range of soil metal(loid) concentrations; indicator plants take up metal(loid)s over a wider range than 'normal' plants and the concentrations in their shoots reflect those of the soil, until phytotoxicity prevents further growth and causes death of the plant; and, accumulator plants are able to actively take up and concentrate metal(loid)s in their above-ground tissues without showing symptoms of toxicity. Within the accumulators are the denominated hyperaccumulators which have a highly specialised physiology permitting them to accumulate extreme amounts of trace metals in their shoots when growing in metal(loid)-enriched substrates (Brooks *et al.*, 1977). These metal(loid)s reach concentrations that would be toxic to most other plant species.

The first plant species documented to accumulate such extreme levels of metal(loid)s in their leaves, already at the end of the 19th century, were *Thlaspi caerulescens* (now renamed as *Noccaea caerulescens* (Meyer, 1973; Al-Shehbaz, 2014)) and *Viola calaminaria* (Baumann, 1885). Later, in the 1940s, it was reported that *Alyssum bertolonii*, growing in the Tuscany region of Italy, contained an extraordinarily high Ni content of about 10000 mg kg^{-1} in its shoot dry matter

(Minguzzi and Vergnano, 1948). However, the specific use of the term "hyperaccumulator" was introduced by Brooks *et al.* (1977) to describe plants containing a Ni concentration of greater than 1000 mg kg^{-1} in dry material. Later on the use of the term was widened to include other elements: the same criteria as Ni was used for Cu (Malaisse *et al.*, 1978), Co (Brooks *et al.*, 1980), Pb (Reeves and Brooks, 1983) and As (Reeves, 2006). While a threshold of 10000 mg kg^{-1} (1% dry weight (DW)) was stipulated for Zn and Mn (Reeves and Brooks, 1983; Baker and Brooks, 1989) and of 100 mg kg^{-1} (0.01% DW) for Se and Cd (Reeves, 2006). Recently these criteria have been considered somewhat conservative and a lower threshold has been proposed for elements such as Co, Cu and Cr (300 mg kg^{-1}; 0.03% DW), or for Zn (3000 mg kg^{-1}; 0.3% DW) (Krämer, 2010; van der Ent *et al.*, 2013).

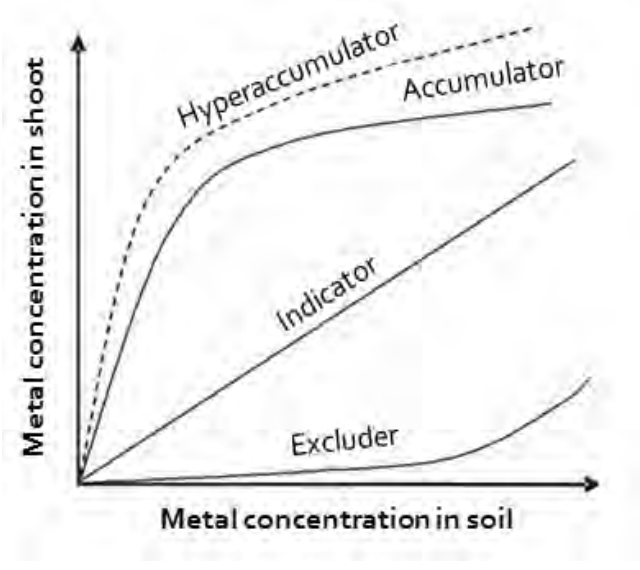


Figure 1.1. Typical plant responses to increasing soil metalloid) concentrations (Baker, 1981).

Although still a topic of much debate, the hyperaccumulation trait is thought to have evolved as a means of reducing competition from other plant species ('elemental allelopathy'), or as a defence mechanism against herbivores and pathogenic microorganisms (Boyd and Martens, 1998; Boyd and Jaffré, 2001; Davis *et al.*, 2001; Boyd, 2007). There are approximately 500 taxa of plants that are known to hyperaccumulate one or more metals or metalloids, and around 450 of these accumulate Ni (Hunt *et al.*, 2014; Pollard *et al.*, 2014; van der Ent *et al.*, 2015). Within the Ni hyperaccumulators the most represented plant families include the Brassicaceae, Euphorbiaceae, Asteraceae, Flacourtiaceae, Buxaceae and

Rubiaceae, although Ni hyperaccumulators are also present in another 30 families distributed throughout the plant kingdom, indicating that the hyperaccumulation trait must have evolved independently several times (Reeves, 2006; Pollard *et al.*, 2014). The Brassicaceae contains 25% of known Ni hyperaccumulators and the largest single clade is found in the section Odontarrhena of the genus *Alyssum*, which contains 48 Ni-hyperaccumulating taxa whose distribution is strongly correlated with the occurrence of ultramafic (serpentine) outcrops around the Mediterranean basin (Brooks, 1987; Krämer, 2010; Pollard *et al.*, 2014). In fact, the vast majority of Ni-hyperaccumulators appear to be serpentine-endemic species (Reeves and Adigüzel, 2008). The Iberian Peninsula hosts two Ni-hyperaccumulating subspecies of *Alyssum serpyllifolium* Desf. (Brassicaceae) which are both serpentine-endemic: *Alyssum serpyllifolium* ssp. *lusitanicum* Dudley and P. Silva (frequently referred as *A. pintodasilvae*) growing at the serpentine outcrops of Barazón (NW Spain) and Trás-os-Montes (NE Portugal) (Pinto da Silva, 1970; Freitas *et al.*, 2004; Díez-Lázaro *et al.*, 2006) and *Alyssum serpyllifolium* ssp. *malacitanum* Rivas Goday (commonly referred as *A. malacitanum*) (López-González 1975) growing at the serpentine outcrops of Sierra Bermeja and Sierra de Aguas (S Spain). The subspecies *A. serpyllifolium* Desf. ssp. *serpyllifolium* (non-hyperaccumulator) is also found growing in limestone-derived soils in NW Spain and S Spain.

The list of Zn hyperaccumulators is much shorter than that of Ni hyperaccumulators, with *c.* 10-20 identified species (Baker *et al.*, 1992; Brooks, 1994). They are mainly found growing on calamine soils, although for some species such as *N. caerulescens* and *Arabidopsis halleri*, non-metallicolous accessions have also been reported (Meerts and Van Isacker, 1997; Bert *et al.*, 2000). Zn hyperaccumulation appears to be concentrated in the genera *Noccaea* and *Arabidopsis* (both within the Brassicaceae family), while just a few species in other families have been found (including members of the *Sedum* genus (Crassulaceae) in Asia) (Baker and Brooks, 1989; Deng *et al.*, 2016; Gupta *et al.*, 2016; Martos *et al.*, 2016). Hyperaccumulation of Cd has been reported for the Zn hyperaccumulators *N. caerulescens*, *A. halleri*, *S. plumbizincicola* and *S. alfredii* (Brown *et al.*, 1995; Küpper *et al.*, 2000; Lombi *et al.*, 2000; Deng *et al.*, 2016) and the Ni hyperaccumulator *Noccaea goesingensis* (Lombi *et al.*, 2000).

Some plant species native to seleniferous soils accumulate Se to levels typically 100-fold higher than other vegetation: upwards of 1000 mg kg^{-1} (DW) and up to 15000 mg kg^{-1} (DW); these are considered Se hyperaccumulators (Beath *et*

al., 1939). Selenium hyperaccumulation has been reported in ~30 species in the families Brassicaceae (*Stanleya*), Fabaceae (*Astragalus*) and Asteraceae (*Xylorhiza*, *Oonopsis*, *Symphyotrichum*) (Rosenfeld and Beath, 1964; Cappa and Pilon-Smits, 2014).

Trace metal contamination in soils

In addition to natural geogenic sources, elevated concentrations of trace metals may be present in the soil due to anthropogenic activities (Table 1.2). Soil contamination is defined by the European Commission (EC) as "the occurrence of pollutants in soil above a certain level causing a deterioration or loss of one or more soil functions" or "the presence of man-made chemicals or other alteration in the natural soil environment"[1]. Trace metals are one of the most frequent soil contaminants (24%) present at polluted sites across Europe (Panagos *et al.*, 2013) and excess TE concentrations has been identified by the EC as one of the eight major threats to European soils, and thus food safety, animal and human health, and ecosystem services (COM, 2002).

Soil is an extremely complex medium which performs vital functions such as biomass production, storage, filtration and transformation of substances. Soil has a role as a habitat and gene pool, serves as a platform for human activities, landscape and heritage and acts as a provider of raw materials. It contains around twice the amount of carbon in the atmosphere and three times the amount found in vegetation (Lorenz, 2013). As its formation is an extremely slow process and supports many vital functions, soil can be considered as an essential and non-renewable resource[2]. Soil generates numerous ecosystem services which are classified as (i) supporting (soil formation, nutrient cycling, habitat), (ii) regulating (regulation of elemental cycles; C sequestration; water purification and storage; adsorption and transformation of pollutants), (iii) provisioning (raw materials and biomass; physical stability and support of plants), and (iv) cultural (heritage sites, archaeological archive) (Lorenz, 2013). Contamination leads to a decline in soil quality and biodiversity, which in turn reduces the soil's capacity to perform ecosystem functions and provide these essential ecosystem services.

The Thematic Strategy for Soil Protections of the European Union (EU) highlights soil contamination amongst the main soil degrading processes together

[1] http://eusoils.jrc.ec.europa.eu/library/themes/contamination/

[2] http://ec.europa.eu/environment/soil/index_en.htm

Table 1.2. Main sources of metal(loid) pollution and their most common uses in industry (adapted from Adriano, 2001).

	Sources of pollution	Uses in the industry
Ni	Mining and smelting, biosolid applications, fertilisers, coal	Electroplating, alloy production and fabrication, Ni-Cd batteries and electronic components, and the preparation of catalysts for hydrogenation of fats and methanation. Stainless steel production, magnetic components, resistance to corrosion, heating elements
Cr	Metallurgical (ferroalloys and nonferrous allows), refractory and chemical industries. Electric furnaces, refractory brik, steel production, coal combustion, fertilisers, waste disposal.	Manufacture of stainless steel, refractory industry, chemical industry (leather tanning, catalysts, pigments, textiles, wood preservatives and toner)
Co		High-grade steels, alloys, superalloys and magnetic alloys, drying agent, pigments glass decoloriser and catalysts in the petroleum industry
Cu	Electrical industry, metallurgical processing, iron and steel production and coal combustion, manure and sewage sludge addition	Wire, fertilisers, bartericides and fungicides and in water putification, feed additive (antibiotics, drugs, chemical compounds, growth promoter agent for disease control)
Cd	Fertilisation, sewage sludge, incineration, fossil fuel, metallurgical, mining and smelter	Alloys, electroplating, pigments, stabilisers for polyvinyl plastics, batteries
Zn	Automobile industry, fertilisation, sewage sludge, mining and smelting	Protective coating to prevent corrosion and alloys such as building, transportation and appliance industries, water delivery industry. Manufacture, ingredient of several household items, fertilisers, textiles, adhesives or wood preservatives
Pb	Mining, smelting, refining, manufacturing, and recycling and disposal of Pb-containing products	Large rechargeable batteries, pigments, rolled and extruded products, cable sheathing, alloys, shot and ammunition and gasoline additives

with erosion, losses in OM, compaction, salinisation, landslides, and soil sealing. The large volumes of industrial, municipal and agricultural wastes and widespread use of chemicals during past decades have left numerous contaminated sites across Europe. Local sources of soil contamination are mainly attributed to an inadequate or unauthorised waste disposal, or to the unsafe handling of dangerous substances within industrial or commercial processes and associated accidents (Panagos *et al.*, 2013). The European Environmental Agency (EEA) estimated 2.5 million sites as potentially contaminated in EU countries and 342000 as contaminated sites, of which approximately 15% have already been remediated[3]. In general, production sectors (60%) contribute more to local soil contamination than service sectors (32%). The main anthropogenic sources of trace metals are associated with present

[3] EEA, http://www.eea.europa.eu/data-and-maps/indicators/progress-in-management-of-contaminated-sites-3/assessment

and former mining activities, foundries, smelters (point sources) and diffuse sources such as piping, combustion of by-products, and traffic related to industrial and human activities (Panagos *et al.*, 2013).

In Spain, 71202 sites have been identified as potentially contaminated while 285 have been identified as contaminated (van Liedekerke *et al.*, 2014). Most European countries have national or regional legislation to regulate local soil contamination but no legal framework has yet been established at the level of the EU. In general, legislation aims at preventing new contamination and setting targets for the remediation of sites where environmental standards have already been exceeded. At a Spanish level, soil contamination was regulated until 2011 by the Law 10/1998 of Residues and the Royal Decree 9/2005 of the 14th of January (BOE no. 15, 18 January 2005). Nowadays, soil contamination is regulated by the Law 5/2013 of Contaminated Soils of the 12th June (BOE no. 140, 12 June 2013) of Contaminated Soils and the Royal Decree 9/2005, both within the *II Plan Nacional de Suelos Contaminados* (2007-2015). These provide a list of potentially soil contaminating activities and the standard criteria on the basis of which a soil is declared as contaminated together with the definition of Generic Values of Reference (NGRs according to the Spanish spelling). NGR is the concentration of a contaminant in soils which does not entail a higher risk than that which is considered as the acceptable maximum for human health and ecosystems. The Royal Decree includes the NGRs for organic contaminants but not for TE and this responsibility is transferred to the regional governments, since metal(loid)s occur naturally in the soils and their background level and toxicity is strongly dependent of the geological substrate. In Galicia, the NGR values for TE are defined in the Soil Contamination Decree 60/2009 of the 6th of February (DOG no. 74, 24 March 2007). In terms of the addition of metal(loid)s to soils through soil amendments and/or fertilisers, the maximum permitted concentrations of TEs in sewage sludge and composts destined for agriculture use are regulated by the Royal Decree 1310/1990 (Ministry of Agriculture 1990) and by the Spanish Law on fertilisers, Royal Decree 824/2005 (Ministry of Agriculture 2005) respectively. However, the use of these waste products or residues as amendments in marginal or degraded lands (such as mine-soils) is not contemplated in current legislation.

Mining activities are amongst the most polluting activities in the EU. The processing of the metallic ores involves several chemical and physical separation steps, such as the crushing and grinding of rocks, and the separation of waste from valuable products (Dudka and Adriano, 1997; Hoskin *et al.*, 2000). The ore

represents only a small fraction of the total volume of mined material; as a result the beneficiation processes generate large volumes of residues. These operations are restricted to relatively small areas, but tailings and rock deposits can cover extensive areas and are the main source of metal(loid) contamination (Salomons, 1995). Although mines are classified on the basis of their predominant product, they may produce large quantities of other toxic elements as co-products. As a result, metal(loid) ore processing usually leads to the multi-elemental contamination of the environment (Dudka and Adriano, 1997). In mine tailings, the main limitations for plant colonisation and growth are the lack of structure, water retention capacity, OM and essential nutrients (Wong, 2003; Ernst, 2005). In addition to the contamination of soils, mining areas are also sources of water and air pollution, arising from surface water runoff, acid mine drainage (which refers to the acidic waters produced by the dissolution of exposed sulphide minerals containing metal(loid)s in the presence of oxygen and water) and dust particles produced by grinding operations or wind erosion from bare soils (Hoskin *et al.*, 2000).

1.2. Soil trace element availability and plant uptake and accumulation

Metal(loid) (bio)availability can be defined as the metal(loid) fraction that can interact with a biological target (Geebelen *et al.*, 2003). Plants acquire nutrients and trace metals from the soil which is in close contact with the roots (defined in 1904 by Hiltner as the rhizosphere). Metal(loid) availability to plants is governed by a pseudo-equilibrium between the elements in the soil solution and in the solid phase (Kidd *et al.*, 2009) and is therefore determined not only by the concentration in the soil solution (from which plants mainly absorb soil elements) but also by the capacity of the solid phase to resupply the element (Bruemmer *et al.*, 1986). In the soil solution elements are present as free uncomplexed ions, ion pairs, ions complexed with organic anions, and ions complexed with organic macromolecules and inorganic colloids. The most important metal(loid) pools in the solid phase include the exchange complex, metal(loid)s complexed by OM, sorbed onto or occluded within oxides and clay minerals, co-precipitated with secondary pedogenic minerals (e.g. Al, Fe, Mn oxides, carbonates and phosphates, sulphides) or as part of the crystal lattices of primary minerals (Adriano, 2001). When the rate of metal(loid) uptake by the plant exceeds its diffusional transport through the pore water its concentration in the soil solution is depleted and this induces resupply from the labile pools. Resupply occurs through the dissociation of complexes present in the solution phase but also via the release of labile

elements associated with the solid phase (e.g. on the exchange complex, complexed by OM) and is determined by the concentration of the element in the labile pool, its diffusional supply and the rate at which it is released from solid phase to solution (Lehto *et al.*, 2006). Adsorbed metal(loid)s are exchangeable with the soil solution while the occluded fraction is (at least temporarily) unavailable for exchange with the soil solution. Finally, the fraction of trace metals which is structurally incorporated into soils minerals is not likely to become plant-available (Adriano, 2001).

Plants mobilise soil nutrients and/or trace metals by actively changing the soil physicochemical properties in the rhizosphere. Plants differ in their capacity to convert non-available forms of (micro)nutrients to available forms according to differences in their root surface area (Sadana *et al.*, 2002), in the composition and amount of root exudates (Rengel, 2002; Jones *et al.*, 2004), or in their associated rhizospheric microbiota (Marschner *et al.*, 2004). Between 10 to 20% of the photosynthetic carbon that roots receive is released as rhizodeposits in the rhizosphere (Singer *et al.*, 2003). These exudates are believed to be implicated in the mobilisation of soil elements through the weathering of minerals (Hinsinger *et al.*, 1993; Jones and Edwards, 1998; Kidd *et al.*, 2001; Barceló and Poschenrieder, 2002). Moreover, root-induced reactions such as chelation, precipitation or changes in pH and redox conditions also influence soil element availability, as well as the activity of plant-associated rhizosphere microorganisms (Uren and Reisenauer, 1988: Tao *et al.*, 2004).

Interactions between plant exudates and N, P or Fe nutrition are well known (Hinsinger, 2001; Jones *et al.*, 2004; Hinsinger *et al.*, 2009; Dotaniya and Meena, 2015). Organic anions (such as citrate, oxalate or malate) are released into the rhizosphere in response to various nutritional stresses including P, Fe and micronutrient deficiency but also under Al toxicity (see reviews by Hocking, 2001; Ryan *et al.*, 2001; Neumann and Römheld, 2007). Organic anions are commonly released from roots in association with protons which results in an acidification of the rhizosphere (Dinkelaker *et al.*, 1989; Hoffland *et al.*, 1989; Neumann *et al.*, 2002). Both, the decrease in pH and the complexing capacity of organic anions can also directly facilitate the mobilisation of sorbed P (Dinkelaker *et al.*, 1989; Hoffland *et al.*, 1989; Ström *et al.*, 2005). Under Fe deficiency, Poaceae are known to secrete phytosiderophores, Fe(III)-solubilising molecules which are able to complex non-soluble ferrous iron and make it available for plant uptake (Römheld and Marschner, 1986; Kobayashi and Nishizawa, 2012). Other molecules, such as fla-

vonoids, act as signal compounds in the establishment of the N_2-fixing symbiosis with rhizobia under deficiency of external N supply (Cesco *et al.*, 2010).

The processes determining nutrient and metal(loid) speciation, distribution and availability are controlled by the combination of soil properties, plant characteristics and interactions with associated plant microorganisms (Jones *et al.*, 2004).

Metal(loid) availability in the rhizosphere of hyperaccumulators

Metal(loid) hyperaccumulating plant species have an extraordinary capacity for trace metal accumulation, and furthering our understanding of the mechanisms controlling metal(loid) availability in the rhizosphere and their subsequent uptake and accumulation in shoots could lead to the development and improvement of remediation methods for metal(loid) contaminated soils using these plants. Root system architecture plays an important role in nutrient uptake, since it determines the rhizosphere volume and plant access to soil elements (Comerford, 2005). Metal(loid) hyperaccumulators have generally been described with shallow root systems (< 0.5m) but they present a high proportion of fine roots which increases the surface area in contact with soil solution and greatly contributes to TE accumulation (Keller *et al.*, 2003; Himmelbauer *et al.*, 2005). Direct root growth towards metal(loid)-rich patches in soil (chemotropism) has been reported in some hyperaccumulating species or ecotypes, for example, root foraging towards Zn and Cd metal(loid)-rich patches has been documented in *N. caerulescens* (Schwartz *et al.*, 2003). However, non-hyperaccumulator plants are also known to alter their root growth to compensate for spatial variability in soil nutrients (which could be co-localised with trace metal(loid)-rich patches) (Jackson and Caldwell, 1996; Robinson, 1996; Bloom *et al.*, 2003; Doussan *et al.*, 2003).

A higher metal(loid) accumulation in hyperaccumulators has been related to a higher expression of metal(loid) transporters at the root surface of these plants compared to non-hyperaccumulators, or to the presence of transporters with different kinetic properties (Pence *et al.*, 2000; Li *et al.*, 2005). Specific transporters for essential elements are well documented (Grotz *et al.*, 1998; Clemens, 2001; Pittman, 2005; Burkhead *et al.*, 2009); and non-essential elements have been shown to enter roots through the same transporters by replacing these essential elements. For instance, As, Cd and Se can respectively replace P, Cu/Fe/Mn/Zn or S (Cataldo *et al.*, 1983; Marschner, 1995) due to their similar valence states and ionic diameter. In addition to membrane transporters, hyperaccumulators can promote metal(loid) accumulation through and increased release of metal

(loid) chaperones and chelators; (i.e. glutathione, phytochelatins, organic acids, histidine or nicotianamine) which can facilitate metal(loid) transport within root cells and plant xylem or serve to increase plant metal(loid) tolerance (Charnock *et al.*, 1996; Salt *et al.*, 1999b; Küpper *et al.*, 2001; Weber *et al.*, 2004; Kramer, 2005; Centofanti *et al.,* 2013).

Soil acidification has also been implicated in the metal(loid) hyperaccumulation process due to the high dependence of metal(loid) availability on pH (Delorme *et al.*, 2001). However, several studies have now shown that the rhizosphere of hyperaccumulators, such as *N. caerulescens* (Knight *et al.*, 1997; Hutchinson *et al.*, 2000; Luo *et al.*, 2000), *A. murale* (Bernal and McGrath, 1994), *A. pintodasilvae* (Kidd *et al.*, 2007), and *N. goesingensis* (Puschenreiter *et al.*, 2003; Wenzel *et al.*, 2003), presents a higher pH than that observed in non-vegetated soil. Moreover, the observed acidification in the rhizosphere of *N. caerulescens* could not account for the increase in Zn uptake by the plant since a similar pH was found in the non-hyperaccumulator *Thlaspi ochroleucum* (McGrath *et al.*, 1997).

Dissolved organic matter (DOM) is one of the most important factors affecting metal(loid) mobility and phytotoxicity in soils. It consists of several types of low molecular weight organic compounds, such as polyphenols, simple aliphatic acids, amino acids and sugar acids (Kaiser *et al.*, 2002). The effect of DOM on metal(loid) availability differs according to the composition and concentration. For example, dissolved organic carbon (DOC) has been shown to immobilise and detoxify metal(loid)s such as Cu (Römkens *et al.*, 1999), and on the contrary but more frequently, to increase trace metal(loid) availability such as Fe or As (Fitz *et al.*, 2003). DOM derived from the rhizosphere of the hyperaccumulating ecotype of *Sedum alfredii* could significantly reduce Cd and Zn sorption and increase their mobility through the formation of soluble DOM–metal(loid) complexes (Li *et al.*, 2011). Wenzel *et al.* (2003) found significantly increased DOC and Ni concentrations in water extracts of *N. goesingensis* rhizosphere soil, whereas exchangeable Ni was depleted due to excessive uptake of Ni by the hyperaccumulator. Through modelling and chemical speciation analysis, these authors suggested that enhanced Ni solubility in the rhizosphere of the hyperaccumulator was driven by the formation of Ni-organic complexes.

Many authors have considered the possibility that hyperaccumulating plants actively mobilise metal(loid)s from non-labile soil metal(loid) pools as a means of increasing the labile concentrations and consequently plant uptake and accumula-

tion (Alford *et al.*, 2010). McGrath *et al.* (1997) found that the mobile fraction of Zn in soils accounted for less than 10% of accumulated Zn in shoots of *N. caerulescens*, while in a closely related non-hyperaccumulating species 55% of the shoot Zn was obtained from the mobile fraction. In another study, a reduction in the residual Ni fraction (bound to silicates) was found in the rhizosphere of *A. pintodasilvae* alongside an increase in the reducible and oxidisable fractions and this was not observed in a non-hyperaccumulator species (Kidd *et al.*, 2007). However, most studies in fact suggest that these plants access the same metal(loid) fractions as non-hyperaccumulators and do not actively mobilise metal(loid)s from less plant-available pools (Salt *et al.*, 2000). More recent studies have suggested that the release of organic ligands (such as citrate) by the roots of hyperaccumulating species could accelerate the weathering process of Ni-bearing phases in the rhizosphere thus maintaining efficient resupply of the labile metal pool and sustaining the elevated Ni uptake capacity of these plants (Boominathan and Doran, 2003; Wenzel *et al.*, 2003; Kidd *et al.*, 2009). It seems more likely that hyperaccumulators do not possess specific metal(loid)-mobilising mechanisms, and that the pronounced depletion in the rhizosphere, caused by the high rate of uptake of the element from the soil solution, induces their replenishment from the less mobile to the mobile pools. This replenishment results in a sustained high concentration in the soil solution (Figure 1.2a). In contrast, in metal(loid)-excluding plants (such as the Poaceae) the opposite would occur, where a low uptake by the plant would shift the equilibrium to the solid phase resulting in a lower re-upply (Figure 1.2b). Root and microbial activity of *N. goesingensis* have been proposed to participate in an enhanced dissolution of Ni-bearing minerals, contributing to a higher Ni uptake (Puschenreiter *et al.*, 2005). Similarly, mineralogical studies showed the presence of smectite in the rhizosphere of *A. pintodasilvae*, which was related to a more intense weathering of Ni-rich ferromagnesium minerals (chlorite, serpentine) and an increase in labile Ni (Kidd *et al.*, 2009). Chardot-Jacques *et al.* (2013) found that the growth of the Ni-hyperaccumulator *Leptoplax emarginata* increased the dissolution of chrysotile (silicate from the serpentine group with low Ni solubility). These authors suggested that the high Ni uptake by the plant causes a decrease in water-soluble Ni which in turn induces chrysotile dissolution. Plant-associated microorganisms are also believed to influence soil metal(loid) availability, and microbial transformation of soil minerals leads to the solubilisation of metal(loid)s alongside essential nutrients. In fact, microorganisms associated with hyperaccumulators have

been implicated in the metal(loid) hyperaccumulation process. Becerra-Castro *et al.* (2013) observed bacterial-promoted dissolution of soil minerals leading to Ni release by siderophore- and organic acid-producing bacterial strains which were isolated from the rhizosphere of Ni-hyperaccumulating subspecies of *A. serpyllifolium*. Both root and microbial activity and their interactions are believed to have a strong influence on the processes involved in metal(loid) replenishment. The influence of plant-associated bacteria on metal(loid) availability and the effectiveness of phytoremediation techniques will be further discussed in the following sections.

Estimating soil metal(loid) availability to plants

A great variety of methods have been proposed for assessing metal(loid) availability in soils (Michel and Ludwig, 2005). The free metal(loid) ion is generally considered as the chemical species which is most mobile and phytoavailable. To predict metal(loid) uptake by plants the free ion activity or the total element concentration in the interstitial soil pore water (or soil solution) is measured. However, free metal(loid) ion activity can only be measured directly in the case of a few species of TEs e.g. using ion-selective electrodes for Cu^{2+}. Speciation in soil pore waters is therefore generally carried out using computer programs such as GEOCHEM. The most common approaches used to estimate the available metal(loid) fraction and correlate this with bioaccumulation in plants include chemical extraction techniques, based on either single or sequential extractions (Michel and Ludwig, 2005; Meers *et al.*, 2007; Menzies *et al.*, 2007; Hass and Fine, 2010). Single extractants commonly used include dilute neutral salts ($NaNO_3$, NH_4NO_3, $CaCl_2$, $Ca(NO_3)_2$), dilute acids(CH_3COOH), and metal(loid) chelant agents (ethylenediaminetetraacetic acid **(**EDTA) or diethylene triamine pentaacetic acid DTPA). Menzies *et al.* (2007) found trace metal(loid) concentrations determined by complexing agents or acid extractants were poorly correlated to plant phytoavailability, while more useful indications of metal(loid) phytoavailability were obtained using neutral salt extractants (such as 0.01 M $CaCl_2$ and 0.1 M $NaNO_3$). The sequential extraction procedure is more related to the geochemical distribution of metal(loid)s in soils: specific chemical extractants (of increasing strength) applied sequentially to the soil sample can theoretically assess TE fractions of different solubility related to mineralogical phases. Limitations of sequential extractions include the possible re-distribution of analytes among phases during extraction; non-selectivity of reagents for target phases;

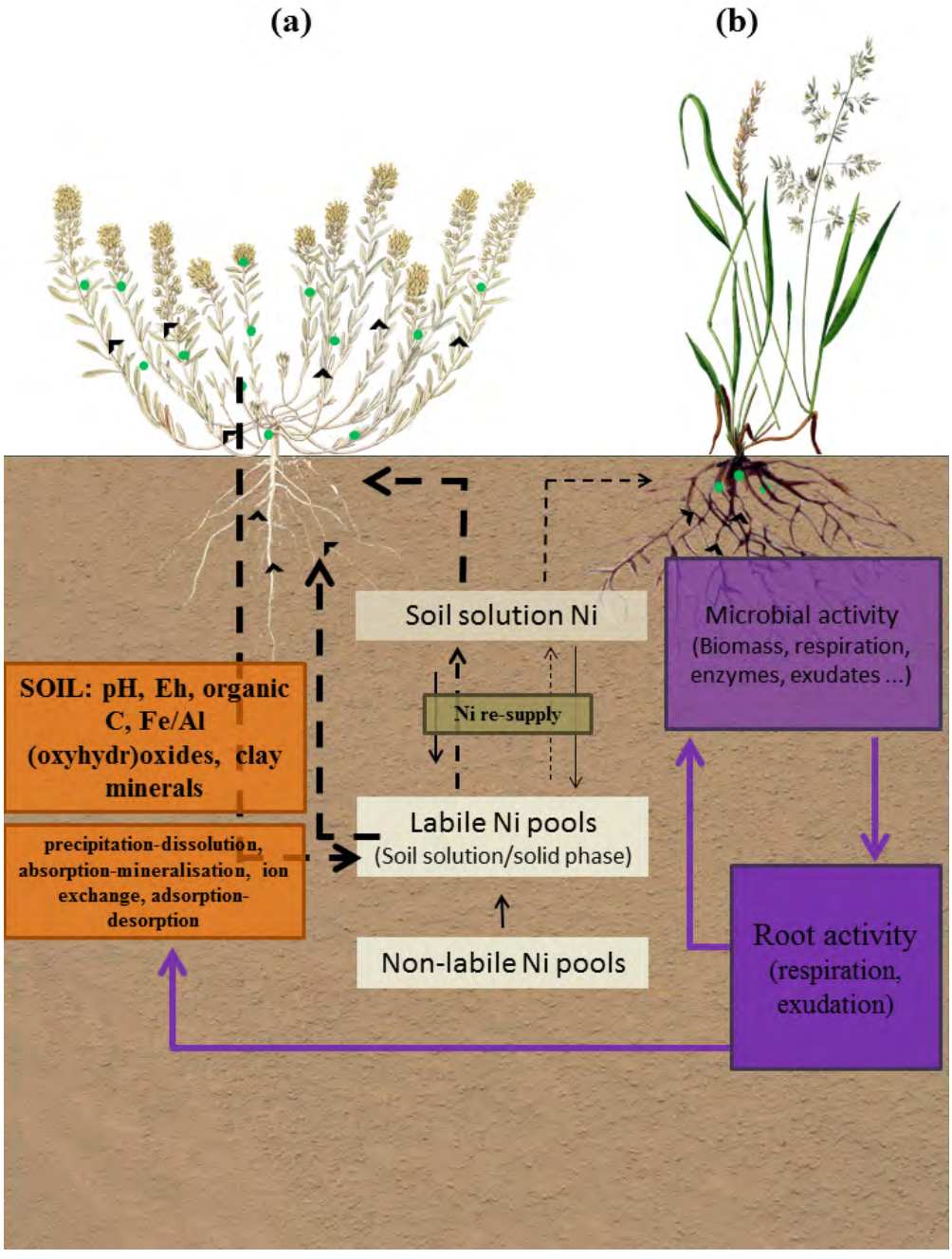


Figure 1.2. Schematic representation of the rhizospheric processes involved in the resupply of metal(loid)s in soil-plant systems of (a) metal(loid)-hyperaccumulators or (b) metal(loid)-excluding plants.

incomplete extraction; precipitation of "new" mineral phases during extraction (Bacon and Davidson, 2008).

Stable isotope dilution procedures have been proposed as alternatives to conventional extraction methods since they take into account the flux of metal(loid)s from the soil solid phase to the soil solution (Hooda *et al.*, 1999; Cattani *et al.*, 2006). The labile trace metal(loid) fraction, estimated through chemical soil extractions, is often relatively poorly correlated with plant metal(loid) uptake. A major limitation to these extractions is that they establish a pseudo-equilibrium between the soil solid phase and the extractant for a given analyte but do not mechanistically mimic element uptake by plants (Kreuzeder *et al.*, 2013). Diffusive Gradients in Thin Films (DGT) was developed as an *in situ* passive sampling technique for measuring the pseudo-equilibrium between the free ion activity, the total concentration in soil solution and the extractable pool in the solid phase, while mimicking uptake by plant roots (Davison and Zhang, 1994; Zhang *et al.*, 2001). Like a plant, the DGT locally lowers the soil solution concentration, inducing resupply from the solid phase. DGT was first introduced as a dynamic speciation tool for measurement of element concentrations in waters (Davison and Zhang, 1994; Zhang and Davison, 1995), but has since then been adopted as an analytical tool to determine the availability of nutrients and contaminants to terrestrial (Zhang *et al.*, 2001; Degryse *et al.*, 2009) and aquatic organisms (Luider *et al.*, 2004), complex dissociation kinetics (Scally *et al.*, 2003) and for 2D chemical imaging of the distribution of labile chemical species in soils and sediments (Davison *et al.*, 1997; Lehto *et al.*, 2012; Santner *et al.*, 2012; Kreuzeder *et al.*, 2013; Hoefer *et al.*, 2015; Valentinuzzi *et al.*, 2015). The DGT-labile solute fraction has been frequently shown to correlate better with plant uptake than that measured by chemical extractants (Davison and Zhang, 2000; Zhang *et al.*, 2001; Degryse *et al.*, 2009; Tandy *et al.*, 2011).

The DGT device typically contains two layers of polyacrylamide gels: a polyacrylamide hydrogel layer with a resin that contains a binding agent and the diffusion layer separated from the soil by a protective filter (Figure 1.3) (Zhang and Davison, 1995). The soil is saturated to its maximum WHC (to ensure a continuous diffusion pathway for metal(loid)s) and brought into contact with the gels containing the binding resin for a known deployment time. During DGT application, the elements diffuse through the diffusive layer towards the resin gel where they are immediately bound, thus creating a gradient between the soil and the resin. Therefore the element concentration at the resin gel (diffusive gel

interface) remains effectively zero, providing a constant zero-sink for the elements which causes a continuous diffusive flux from the soil solution to the gel sampler. At the end of the deployment time, the resin gel is eluted and analysed to determine the amount of element taken up (Santner *et al.*, 2015).

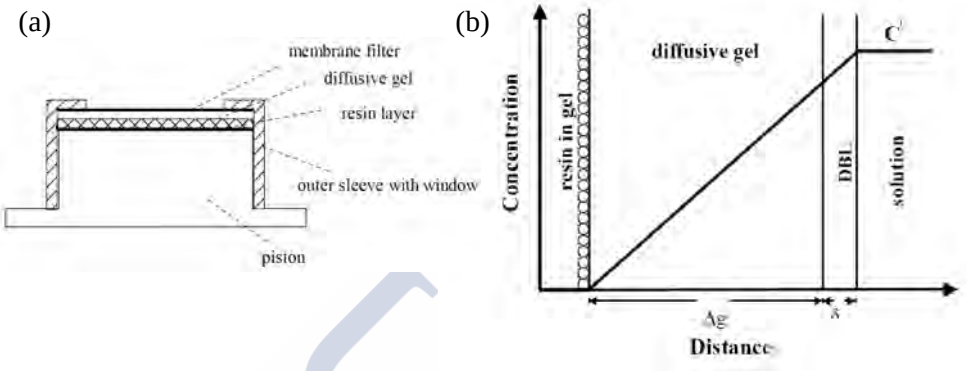


Figure 1.3. Schematic representation of DGT device (a) and (b) schematic cross-section through a DGT device in contact with solution, showing the steady-state concentration gradient. DBL (diffusive boundary layer), C´ (concentration in the soil solution), Δg (thickness of diffusive gel), and δ (thickness of the DBL) (from Zhang and Davison (2006)).

The analytes which can be measured by DGT are determined by the binding agent used. The binding agent for the first DGT was Chelex resin (Davison and Zhang, 1994; Zhang and Davison, 1995) containing paired iminodiacetate ions which act as chelating groups that bind polyvalent metal(loid) ions. Later on, a ferrihydrite gel with strong affinity for some anions (e.g. phosphate, arsenate) was used to measure labile phosphate (Zhang *et al.*, 1998), and silver iodide was included in the gel to measure sulphide (Teasdale *et al.*, 1999). More recently, a binding gel based on zirconium-hydroxide was developed for the simultaneous measurement of cations and anions (Ding *et al.*, 2010; Kreuzeder *et al.*, 2013; Sun *et al.*, 2013).

A key point in this technique is that the DGT continuously removes and accumulates the target elements. The depletion of elements in the soil solution at the interface of the DGT device results in a perturbation of the equilibrium in the soil system. This induces diffusion from further away in the soil solution towards the area of depletion and desorption of sorbed elements (complexes in the soil solution and in the solid phase) for re-establishing the equilibrium state. Consequently all species that are labile, i.e. able to dissociate sufficiently rapidly

to contribute to the flux of the device, will be measured (Zhang and Davison, 2000).

Figure 1.4 illustrates how the extent of depletion in the soil solution depends on the rate of release of the element from the solid phase. When the rate of release from the solid phase is fast, this supply effectively buffers the element concentration in soil solution and very little depletion occurs (Figure 1.4b). A small depletion in the solution would even occur if elements in the solid phase adjacent to the device are consumed. When the rate of release from the solid phase is very slow, there is little supply from this source, and element supply will depend on the diffusion of labile species in solution. In this case, the elements in solution adjacent to the DGT device are quickly depleted, causing a fast decline in the fluxes to the DGT device (Figure 1.4a). The intermediate case occurs when elements are released from the solid phase at a high rate, but insufficient to fully sustain the concentration in solution adjacent to the device (Figure 1.4c) (Zhang and Davison, 2006).

The DGT technique can be used to determine the effective concentration (C_E) of elements in soil solution, and C_E is based on both the soil solution concentration and its supply from the solid soil phase (Zhang *et al.*, 2001). Plant element concentrations have been found to be linearly related and highly correlated with C_E across a wide range of soil types, while nonlinear and more scattered relationships are obtained with free metal(loid) activity, total soil solution concentrations or chemical extractions (e.g. EDTA) (Davison and Zhang, 2000; Nowack *et al.*, 2004; Tandy *et al.*, 2011; Zhang *et al.*, 2004; Zhang *et al.*, 2001). These results demonstrate that the kinetically labile solid phase pool of elements plays an important role in plant uptake and is included in the DGT measurement (Zhang *et al.*, 2001).

DIFS (DGT induced fluxes in sediments and soils)

DGT measurements can be incorporated into numerical models to estimate the parameters quantifying the resupply of metal(loid)s from the solid phase to the soil solution, such as the equilibrium ratio between sorbed and dissolved concentrations (K_D) or the rate of release of sorbed fractions (T_C) (Ernstberger *et al.*, 2002; Ernstberger *et al.*, 2005; Harper *et al.*, 1998). Harper *et al.* (1998) developed a one dimension numerical model, 1D-DIFS (DGT Induced Fluxes in Sediments and Soils), to quantify the processes influencing elemental flux into the DGT sink. The DIFS model describes the interaction of DGT with soil and estimates the

proportion of elements in the solid phase (K_D) which includes all species adsorbed to the solid phase that can desorbed and measured by DGT; as well as the response times with which the equilibrium between the solid and solution phase concentrations takes place (*T*c) (Ernstberger *et al.*, 2002; Ernstberger *et al.*, 2005). An improved version of the DIFS model, working in two dimensions (2D DIFS), was developed by Sochaczewski *et al.* (2007) permitting a more accurate determination of the K_D and T_C parameters.

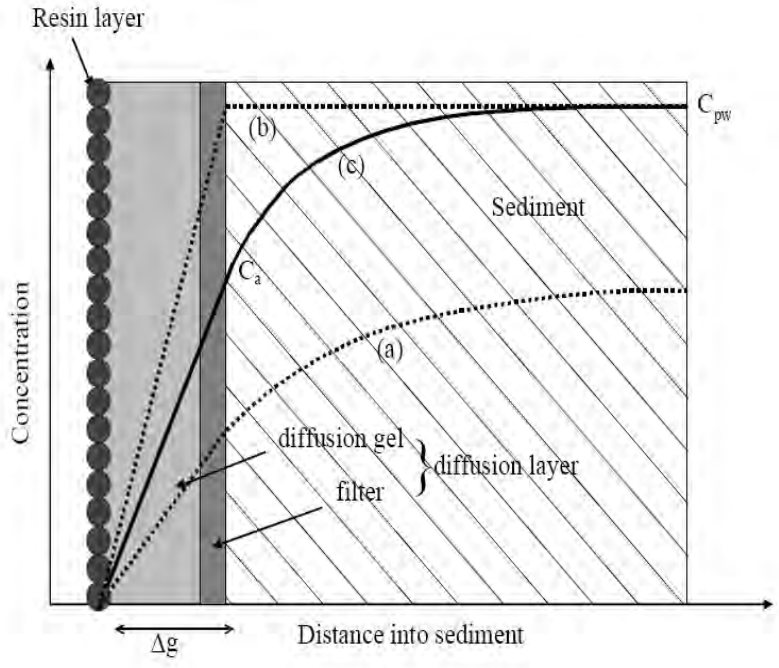


Figure 1.4. Schematic representation of a cross section through a DGT device in contact with a soil or sediment. Pseudo steady-state concentration gradients are illustrated for three cases: (a) unsustained, (b) sustained and (c) the general or partially sustained case. C_a is the interfacial pore water concentration between the sediment and DGT device.

The DIFS model requires the input of several parameters which influence the rate of diffusion of elements such as the diffusion coefficient of the element in water (Do), tortuosity (Θ^2), soil particle concentration (Pc), mass of accumulated elements in the resin gel (M), time of deployment (T) and the R value. The R value is calculated as the ratio of C_{DGT} to the initial measured soil solution concentration (Csol) and is governed by the quantity of elements available for supply from the solid phase and rate of this resupply and is dependent on the deployment time and the extent of depletion (Lehto *et al.*, 2008). For example, R values equal to 1 indicate a continuous resupply from the solid phase to the soil solution while

values lower than 0.1 indicate that resupply is low and there is limited mobilisation from the solid phase after depletion in the soil solution (Conesa *et al.*, 2010). The DIFS model quantifies the dependence of the experimental R (obtained from different deployment times) on resupply of trace metals by quantifying the distribution coefficient between the solid and solution phases (K_D) and the adsorption/ desorption kinetics (T_C) (Ernstberger *et al.*, 2002; Lehto *et al.*, 2008).

Three major sets of conditions that determine the types of soil response to local depletion have been described (Figure 1.4) (Harper *et al.*, 2000; Sochaczewski *et al.*, 2007): (b) sustained case: while dissolved species diffuse towards the DGT sink from the sediment/soil, there is fast resupply from the solid phase that has sufficient capacity to maintain this supply over the time of interest (large T_C, K_D close to zero); (c) partial case: resupply from the solid phase exists, but it is insufficient to sustain the demand by the DGT device at its maximum value (large K_D and medium T_C, or medium K_D and Tc); (a) diffusive case: the capacity of the media (K_D) is very small or the response time of the sediment is very large (T_C) so that there is no effective resupply to the dissolved phase (small T_C and large K_D) (Sochaczewski *et al.*, 2007).

1.3. Gentle remediation options targeting trace element-contaminated land

Remediation of contaminated soils is mainly based on the so-called "traditional" techniques, such as excavation and off-site disposal, which account for about one third of implemented management measures (van Liedekerke *et al.*, 2014). However, in the last three decades an increase in the use of Gentle soil Remediation Options (GRO) has been documented, which include *in-situ* contaminant stabilisation and plant based options (phytoremediation). Phytoremediaton techniques are considered to be cost-effective options and less invasive than conventional civil engineering techniques (Mench *et al.*, 2009; Vangronsveld *et al.*, 2009; Mench *et al.*, 2010; Greenland, 2014). These *green techniques* have been developed to target both organic contaminants and TEs and include the following processes (Wenzel *et al.*, 1999; Salt *et al.*, 1998; Pilon-Smits, 2005; Chaney *et al.*, 2007; Kidd *et al.*, 2009):

-Phytostabilisation uses metal(loid)-excluding plants for the *in-situ* stabilisation of metal(loid)s in soils usually in combination with soil amendments (*aided phytostabilisation*) thus reducing their bioavailability and transfer to the food chain. Phytostabilisation techniques have been suggested to be the more reasonable option for contaminated areas covering large areas and with a high soil metal

(loid) availability (Schwitzguébel, 2014). This technology does not lead to the actual clean-up of the soil, but reduces the potential toxic effects of metal(loid)s in the environment.

-*In situ* stabilisation and phytoexclusion aims to reduce pollutant bioavailability immobilising or binding them to the soil matrix through the incorporation into the soil of organic or inorganic compounds, singly or in combination, to prevent the excessive uptake of essential elements and non-essential contaminants into the food chain. Phytoexclusion is the implementation of a stable vegetation cover using excluder crop plants which do not accumulate contaminants in the harvestable plant biomass can be combined with *in situ* immobilisation.

-Phytoextraction involves the cultivation of tolerant plants that concentrate soil contaminants in their aboveground tissues. At the end of the growth period, plant biomass is harvested and can be burned to produce metal(loid)-enriched ash or "bio-ore" (the process is then known as phytomining). The energy gained from biomass combustion can support the profitability of the technology. Bio-ores can also be used for metal(loid) recovery (smelting, pytometallurgy). When aided by use of soil amendments, the technique is termed aided phytoextraction.

-Phyto(rhizo)volatilisation employs plants and associated rhizosphere microorganisms to transform pollutants into volatile compounds that are then released into the atmosphere.

-Phyto(rhizo)degradation refers to the use of the metabolic capabilities of plants and rhizosphere microorganisms to uptake, store and/or degrade organic pollutants.

Phytostabilisation and phytoextraction are the most common techniques applied to TE-contaminated sites, and both aim to decrease the labile metal(loid) pool in soils which interacts directly with living organisms. When the phytoextracted metal(loid) can be recovered and commercialised with an economical gain, the technique receives the name of phytomining (Li *et al.*, 2003a; Chaney *et al.*, 2007; Tang *et al.*, 2012). A major advantage is their lower cost as compared to the other more conventional remediation techniques (especially when these involve *ex situ* treatment). Moreover, GRO can also provide a range of additional economic (e.g. biomass generation), social (e.g. leisure and recreation) and environmental (e.g. C sequestration, water filtration and drainage management, restoration of plant, microbial and animal communities) benefits (which are encompassed in the generic term "ecosystem services"). On the other hand, the major limiting factor is the time taken to reduce the "total" TE concentrations to below regulatory limits.

This has led to intensive discussions, in particular, about the viability of phytoextraction (Robinson *et al.*, 2009). The factor determining the duration of phytoextraction is the mass of TEs removed by the crop per unit of time (years) compared to the mass of TE in the soil. A decline in plant uptake as soil TE concentrations decline leads to further increases in the time needed for clean-up (Robinson *et al.*, 2009). Phytoextraction is only considered applicable in low to moderately contaminated soils, and its effectiveness can also be reduced by the limited rooting depth of plants and the presence of contaminant mixtures.

The success of GRO is highly dependent on the solubility and speciation of TE in soil, as well as their tolerance and uptake by plants. Adequate plant selection for each technique will be vital. In phytoextraction-related technologies a high uptake of specific TE into the shoots is desirable, while successful phytostabilisation requires that TE be excluded from the aerial portions. Plant-microbial-soil interactions in the rhizosphere will affect TE mobility and availability, and these can be manipulated to optimise the processes operating in GRO. For example, the development of methods for modifying TE bioavailability could have a positive impact on phytoremediation efficiency. Increasing contaminant bioavailability would improve processes such as phytoextraction, whereas the use of amendments which reduce contaminant bioavailability could substantially improve phytostabilisation (Wenzel, 2009). During the last decade, agronomic and biotechnological methods have been developed to optimise the effectiveness and success of GRO. Some of these methods which are suitable for the remediation of trace metal-contaminated sites are described below. Finally, the number of bench-scale or greenhouse-based studies by far exceeds the number of field-scale evaluations of GRO and there is a clear need for field trials to evaluate their performance under realistic conditions (Mench *et al.*, 2009; Vangronsveld *et al.*, 2009; Mench *et al.*, 2010; Kidd *et al.*, 2015).

1. 4. Improving efficiency of Gentle Remediation Options

Selection of adequate plant species for implementing gentle remediation options

Metal(loid) toxicity can severely limit the performance and establishment of the plants, and the selection of TE-tolerant plant species is therefore vital for the successful implementation of GRO in metalliferous sites. However, plants must also tolerate numerous additional abiotic and biotic stress factors, such as water and nutrient deficiency, soil acidity or salinity, soil erosion or compaction, flooding, herbivory or pests.

Natural metal(loid)-enriched areas (such as ultramafic regions) or metal (loid)-contaminated sites (such as mine tailings and spoils) are important sources of metal(loid)-tolerant plant genotypes. Despite the unfavourable growth conditions at these sites, several metallophyte plants have evolved mechanisms to tolerate these conditions and are able to colonise these types of substrates (Batty, 2005; Mendez and Maier, 2008; Whiting *et al.*, 2004). Within the same plant species various ecotypes, cultivars, varieties or clones can differ greatly in their response to the presence of contaminants (Vyslouzilova *et al.*, 2003; Marmiroli *et al.*, 2011; Ruttens *et al.*, 2011). The appropriate plant species will depend on the remediation option to be used. For phytoextraction plants must be able to tolerate and accumulate high concentrations of TEs in their harvestable parts and have a reasonably high biomass production.

One relevant option is to use TE-hyperaccumulators, and one argument in favour of their use is the possible recuperation of TE from metal(loid)-rich biomass. Due to the extreme ability of hyperaccumulators to accumulate and concentrate metal(loid)s in their aerial parts (harvestable biomass), these plants have been described as good candidates for phytoextraction techniques. At present, Ni and Co present the highest prices on the market (average in \$USD kg^{-1}: 5.5 for Cu, 2.0 for Zn, 11.4 for Ni, 1.8 for Pb and 31.1 for Co at the London Metal Exchange (LME); July 2015); however, due to the high number and ample distribution of Ni-hyperaccumulators, Ni seems to be the most appropriate element for phytomining (Chaney *et al.*, 2014). The cultivation of hyperaccumulator plants as part of phytomining strategies on mineral wastes (metal(loid)-contaminated soils or residues) or natural metal(loid)-rich soils (sub-economic ores such as ultramafic soils) can generate Ni *bio-ores* (i.e. plant ashes) with up to 25% Ni, which is significantly higher than that present in current lateritic ores (< 1.5%) or normal Ni -ores (approx. 3%) where conventional mining processes are considered economically unviable (Chaney, 1983; Chaney *et al.*, 1998). Moreover, this *bio-ore* is free of Fe, Mn and Mg silicates present in the soil matrix which are known to increase the costs of metal(loid) extraction (Chaney *et al.*, 2010; Koppolu *et al.*, 2003; van der Ent *et al.*, 2015; Zhang *et al.*, 2014).

Phytomining of Ni has been successfully demonstrated in several studies carried out at a field scale (Figure 1.5). Li *et al.* (2003b) reported a biomass production of *A. murale* and *A. corsicum* in field trials of up to 20 t ha^{-1}. With a shoot Ni concentration of more than 20,000 mg kg^{-1} the total amount of phytoextracted Ni was 400 kg ha^{-1}, resulting in a net annual benefit of \$1749 ha^{-1} (with a Ni price

of \$7.33 kg^{-1} Ni and assuming costs of production of \$1000 ha^{-1}). Chaney *et al.*, (2007) reported economic returns of \$16000 ha^{-1} from phytomining processes using *Alyssum* species (at the time of this study the value of Ni was more than \$40 kg^{-1}). Successful phytomining trials have also been reported in Europe, for example, Bani *et al.* (2015) showed that native populations of *A. murale* in Albania could phytoextract an economically-viable crop of Ni after incorporating inorganic fertilisation. These authors achieved a biomass production of 9 t ha^{-1} with a Ni concentration of 11.5 g kg^{-1} which resulted in a total amount of phytoextracted Ni of 105 kg Ni ha^{-1} per year. The estimated costs of the phytomining cycle were around \$1000 ha^{-1} yr^{-1} and, based on the price of Ni at the time of the study (\$10 kg^{-1}; London Metal Exchange on July 2014) the net return was calculated as \$ 1055 ha^{-1} yr^{-1}. These calculations were based on prices of gross Ni metal, however, other Ni products (such as Ni salts, $Ni(NH_4)_2(SO_4)_2.6H_20$) with a high purity and higher value and which can be easily obtained from the biomass of *A. murale*, would substantially increase the profit of the crop (Barbaroux *et al.*, 2012). Other Ni-hyperaccumulating genera such as *Leptoplax* or *Bornmuellera* have also been suggested as potential candidates for Ni recovery (Li *et al.*, 2003a; Zhang *et al.*, 2014).

Studies evaluating the use of metal(loid) hyperaccumulators to phytoextract elements other than Ni, such as, Zn, Cd or As, from contaminated sites can be found in the literature. A field experiment evaluating the use of *N. caerulescens* (Viviez) to phytoextract Cd and Zn from an agricultural soil amended with TE-rich-sewage sludge addition found two successive crops simultaneously extracted about 9% of the total Cd and 7% of the total Zn (Schwartz *et al.*, 2003). Similarly, the same hyperaccumulator extracted up to 5% of total Cd and 11% of total Zn from an industrial soil affected by a former coking plant, and the available Zn fraction (NH_4NO_3-extractable) was reduced by up to 70% (Schwartz *et al.*, 2003). The As-hyperaccumulator *Pteris vittata* decreased total As concentrations in contaminated paddy soils by up to 11.4% after 9 months of growth, while As pore water concentrations decreased up to 77%. Shoot As concentrations in *P. vitatta* ranged from 45 to 206 mg kg^{-1}, which are close to or above the threshold concentrations given for As hyperaccumulation (Ye *et al.*, 2011). Recently, van der Ent *et al.* (2013) suggested the potential for phytomining of rare elements, such as gold (Au) or rare earth elements (REE) such as cerium (Ce) or lanthanum (La)).

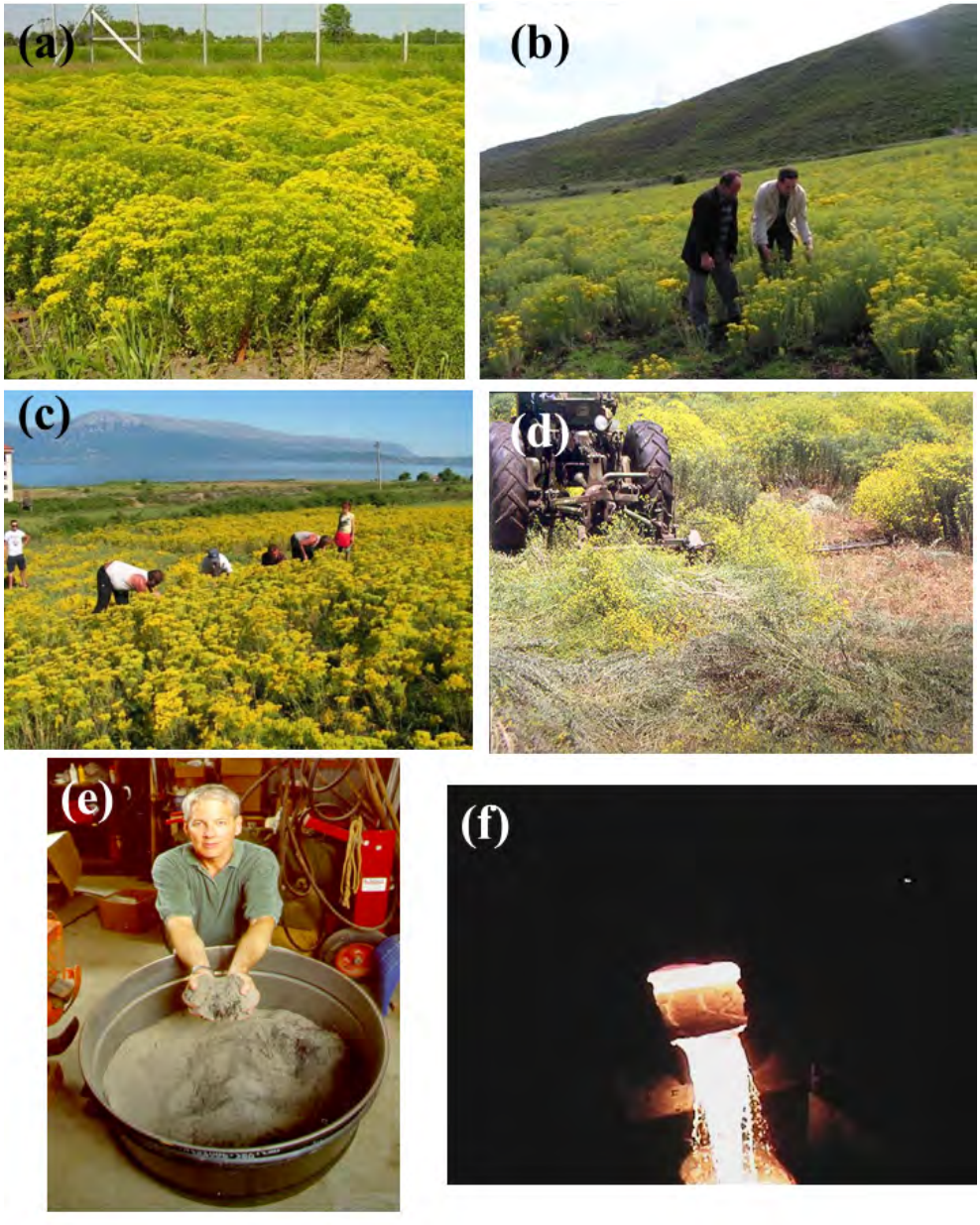


Figure 1.5. Phytomining projects carried out at (a) Refinery field at Port Colborne, Ontatio; *Alyssum corsicum* (b-c) Prrenjas / Pojskë (Albania) *Alyssum murale* (d) harvesting (e) combustion to recover biomass energy and Ni-rich ash and (e) recovery of Ni from ash. *Photos courtesy of Aida Bani and Rufus L. Chaney*

However, to date only Ni phytomining has been proven to be economically feasible (Chaney *et al.*, 2007). The price of metals, such as Zn, on the world market is at present too low to warrant Zn recovery from contaminated soil. On the other hand, Zn enriched biomass obtained during phytoextraction can be used to produce Lewis acid catalysts for organic chemistry, thus providing an economic incentive to the process. Nonetheless, the practical application of hyperaccumulators continues to be limited by the low biomass production of most of these species (e.g. *N. caerulescens*) and the high number of cropping cycles required for clean-up (if the objective is to reduce total TE concentrations in soils). Additional limiting factors include the absence of commercially available seeds, their sensibility to the presence of metal(loid)s other than the hyperaccumulated, lack of knowledge related to their cultivation, climate needs or competition with other metal(loid)-tolerant plants (Kidd *et al.*, 2015).

High-biomass crops and woody plants are recognised as viable alternatives to hyperaccumulators for phytoextraction of TEs (particularly Cd, Se and Zn) if they show moderate-high bioconcentration factors (BCF) and high shoot yields. Both high biomass annual plants and fast growing trees can be easily cultivated using well established agronomic practices (Ghosh and Singh, 2005; Meers *et al.*, 2005; Solhi *et al.*, 2005). Extensive work has been done on the trace metal(loid) uptake capacity of high biomass crop plants, such as Indian mustard (*Brassica juncea*), sunflower (*Helianthus annuus*), and maize (*Zea mays*) (Cui *et al.*, 2004; Turgut *et al.*, 2004; Szabó and Fodor, 2006). High biomass trees such as willows (*Salix* spp.) and poplars (*Populus* spp.) have great potential in furthering efforts to develop phytoextraction (Liphadzi *et al.*, 2003; Vervaeke *et al.*, 2003). Fast growing trees are ideal plant species due to their extensive root systems, rapid growth, large biomass production and easy harvesting with subsequent re-sprouting (Peuke and Rennenberg, 2005). Zhuang *et al.* (2007) compared the phytoextraction efficiency of two hyperaccumulator species with six high-biomass plant (sub)species. Although metal(loid) accumulation was higher in the hyperaccumulators (*Viola baoshanensis* and *Sedum alfredii*), the high biomass production of the non-hyperaccumulator *Rumex ciprus* (20 t ha^{-1}) compensated for its moderate metal(loid) uptake and metal(loid) removal from soils was similar for both plant types. *In vitro* breeding (cell and callus tissue culturing on metal(loid) spiked media) and chemical mutagenesis can significantly improve the metal(loid) tolerance and phytostabilisation or phytoextraction capacity of high yielding annual crops such as tobacco or sunflower (Guadagnini, 2000; Herzig *et al.*, 2005;

Nehnevajova *et al.*, 2007; Nehnevajova *et al.*, 2009). These non-genetically modified plants can be directly tested for their metal(loid) extraction potential under real field conditions without any legal restrictions (Herzig *et al.*, 2014). Sunflower accumulates moderate amounts of metal(loid)s from contaminated soil and water, but their high biomass production makes them interesting for phytoextraction (Lombi *et al.*, 1998; Madejón *et al.*, 2003; Nehnevajova *et al.*, 2005). Furthermore, a negligible metal(loid) concentration in sunflower seeds and oil limits the risk of food chain contamination and should allow the use of oil for biofuels (Kashem and Singh, 2002; Madejón *et al.*, 2003). Herzig *et al.* (2014) estimated a remediation time of between 10 to 46 years for a smelter-affected site with high Zn contamination (16 mg kg $NaNO_3$-extractable Zn) using rotations of improved cultivars of sunflower (accumulating up to 346 mg Zn kg^{-1} and biomass production of up to 26.8 t ha^{-1}) and tobacco (accumulating up to 617 mg Zn kg^{-1} and biomass production of up to 37.5 t ha^{-1}). Delplanque *et al.* (2013) calculated a remediation time 41 years to decrease the total soil Cd concentration from 2.39 mg kg^{-1} to 2 mg kg^{-1} (threshold concentration according to the French regulation) using *Salix schwerinii x Salix viminalis* "Tora" (a well-known Cd accumulator) if both stems and leaves were harvested. The remediation time increased to up to 62 years if only stems were harvested. The long times needed for soil clean-up indicate that the time-scale for Cd phytoextraction could only be acceptable if the remediation process is combined with biomass production for bioenergy or feedstock. In the latter study, the authors also calculated the removal efficiency of Cd according to the extractable fraction, in this case, clean-up time ranged from 1 to 2 years. This process is known as *bioavailable metal(loid) stripping* and several authors have argued in favour of this rather than targeting reductions in the total metal(loid) contamination (Herzig *et al.*, 2014). However, current legislation in most of European countries and initial regulations at a European level for the identification of pollutants thresholds are still based on total metal(loid) concentrations (Bachmann *et al.*, 1999; EC, 2006). Figure 1.6 shows examples of field trials implementing phytoextraction techniques in Europe.

Phytostabilisation often combines re-vegetation with metal(loid)-excluding plants and the incorporation of inorganic and/or organic amendments to improve soil physical, chemical and biological properties (Vangronsveld *et al.*, 1996; Vangronsveld and Cunningham, 1998; Barrutia *et al.*, 2011). Plants

help to stabilise less mobile metal(loid)s (e.g. Cu, Pb) in the soil through uptake and accumulation by the roots, adsorption on root surfaces or precipitation in the rhizosphere (French *et al.*, 2006; Van Nevel *et al.*, 2007; Mendez and Maier, 2008). Plants can alter the chemical form of contaminants by changing the soil environment (e.g., pH, redox potential) around plant roots. Plant-associated microorganisms, which are present in the rhizosphere (bacteria and mycorrhiza) also play an important role in these processes. The role of soil amendments or plant associated bacteria in phytostabilisation techniques will be discussed further in the following sections. Suitable plants for application in phytostabilisation should be adapted to the site edaphic and environmental conditions but also present a reduced transport of metal(loid)s to aerial plant parts. For effective phytostabilisation the bioconcentration factor (BCF) should be < 1 and the translocation factor (TF) or shoot:root ratio < 1. The BCF is defined as the ratio of the concentration of metal(loid) in plant tissues to the initial total (or available) fraction of metal(loid) in soils.

In Europe, metallicolous populations of metal(loid)-excluding grasses, such as *Agrostis capillaris*, *A. gigantea* or *Festuca rubra*, have shown a good ability to colonise metal(loid)-contaminated soils and have been successfully applied in phytostabilisation techniques (Mench *et al.*, 2010). Commercially available cultivars (especially within the genera *Festuca* or *Agrostis*) have also shown promising results in terms of establishment and metal(loid) mobility reduction (Alvarenga *et al.*, 2014). Shrubs (such as *Erica cinerea*, *Calluna vulgaris* and *Ulex europeaus*) or ferns (such as *Pteridium aquilinum*) have been found growing in abandoned Pb/Zn mining areas in the north of the Iberian Peninsula and characterised for a metal(loid) excluder phenotype (Barrutia *et al.*, 2011; Becerra-Castro *et al.*, 2012). The use of N_2-fixing species (such as *Ulex* sp.) is beneficial since they are able to cope with the low soil N content and also to increase this property. Metal(loid)-excluding clones of *Salix* spp. or *Populus* spp. have been shown to achieve high yields at TE-contaminated sites (French *et al.*, 2006)). Furthermore, woody tree species can be combined with SRC for bioenergy purposes. Table 1.3 lists examples from the literature of studies assessing various woody crops for application in GRO (mainly as short rotation coppice). French *et al.* (2006) initiated long-term phytostabilisation trials in former landfill sites in the UK, which had received domestic, industrial and chemical wastes in the 1960s and 1970s using *Salix*, *Populus* and *Alnus* spp.



Figure 1.6. Field–scale phytoextraction trials in (a-b) Soil preparation and harvesting of the SRC plantation at the Lommel site (Belgium), (c-d) sunflower and tobacco growing at the Biogeco site. (e-f) Sunflower-tobacco-maize rotations established at the Bettwiesen site in Switzerland

Willow or poplar SRC combined with phytoextraction and phytostabilisation can reach biomass production levels similar to those obtained in SRC on non-contaminated soils. French *et al.* (2006) obtained a biomass production of <2 to 9 t ha^{-1} yr^{-1} in brownfields in the UK, which is close to the indicative 10-15 t ha^{-1} yr^{-1} expected nowadays in well-managed fields (Riddell-Black *et al.*, 1997; Kidd *et al.*, 2015) and even higher than the biomass yield which is expected to give a positive economic return in the UK (8-10 t ha^{-1} yr^{-1}, Mitchell *et al.* (1999)). Even higher productivity was obtained in an eight-year phytoextraction field experiment of a SRC plantation of willow and poplar on contaminated agricultural land in Krummenhennersdorf (Saxony (Germany)), with average shoot yields of up to 16 t DM ha^{-1} yr^{-1} (and a range between 6 to 16 t DM ha^{-1} yr^{-1}) (Röhricht and Ruscher, 2009). Figure 1.7 shows examples of field trials implementing phytostabilisation techniques in Europe.

Soil amendments and fertilisation regimes for modifying trace element availability and optimising plant growth

Soil amendments can strongly reduce the availability of TE to plants, thus reducing phytotoxicity and facilitating the revegetation of contaminated sites during (aided) phytostabilisation. Commonly used amendments include liming agents, phosphates (H_3PO_4, triple calcium phosphate, hydroxyapatite, phosphate rock), Fe/Mn oxyhydroxides, organic materials (i.e. biosolids, sludge, or composts), natural and synthetic zeolites, cyclonic and fly ashes and steel shots (see reviews by Mench *et al.*, 1998; Vangronsveld *et al.*, 2000b; Adriano *et al.*, 2004; Ruttens *et al.*, 2006b; Kumpiene *et al.*, 2008; Bolan *et al.*, 2014). These amendments can reduce metal(loid) solubility by promoting the formation of insoluble precipitates or by increasing soil binding capacity. In general terms, retention of charged metal(loid) species by soil components can be through electrostatic attraction (non-specific adsorption) or chemical bond formation between the ions and soil surface (Sposito, 1984; Li *et al.*, 2006; Zenteno *et al.*, 2013). In addition to reducing TE bioavailability the incorporation of effective amendments can restore soil quality by balancing pH, adding OM, increasing WHC, re-establishing microbial communities, and alleviating compaction.

Numerous experiments (both bench- and field-scale) reporting amendment-induced reductions in soil metal(loid) availability and enhanced plant establishment and growth in TE-contaminated soils are described in the literature (see reviews by Vangronsveld *et al.*, 2009; Mench *et al.*, 2010; Bolan *et al.*, 2014).

Table 1.3. Examples from the literature of studies assessing various woody crops for application in GRO (mainly as short rotation coppice)

Site description	Plant species	Soil management	Type of remediation technique	Pollutants	Experiment duration	References
Dredged sediment disposal site was created in Deûle-mont (Northern France)	Short rotation coppice (SRC) of three willow clones (*Salix viminalis* 'Tora', *S. viminalis* 'Jorr' and *S. schwerinii* x *S. viminalis* 'Björn')		Phytoextraction combined with bioenergy production	As, Cd, Pb, Zn, Cu	8 years	Delplanque *et al.* (2013)
Highly contaminated land adjacent to a metal refinery in Walsall (England)	*Betula pendula*, *Alnus cordata*, *A. incana*, *A. glutinosa*, *Crataegus monogyna* and *S. caprea*	Residues of sugar beet washings and lime	Phytoextraction	Cu, Zn, Cd, Ni, Pb, Cr	2 years	Dickinson (2000)
Contaminated sludge damp into the Guadiamar river at the Aznalcóllar site (Spain)	*Populus alba, Tamarix africana, Fraxinus angustifolia, S. atrocinerea, Quercus ilex subsp. ballota, Olea europaea var. sylvestris, Phillyrea angustifolia, Pistacia lentiscus, Rosmarinus officinalis* and *Retama sphaerocarpa.*	OM and Ca-rich amendments	Phytostabilisation	As, Cd, Cu, Pb, Tl, Zn	6 years	Domínguez *et al.* (2008)
Five brownfield sites in England (former land uses: land-fill, industrial waste, sewage sludge)	*Salix* spp., *Populus* spp., *Alnus* spp., *Betula* spp. and *Larix* spp.	Glyphosate weed-control treatment	Phytoextraction / Phytostabilisation	Cd, Zn, Cu, Ni, Pb, As	7 years	French *et al.* (2006)
Pb/Zn smelter at Arnoldstein (Austria)	Barley, *Plantago lanceolata*, *Holcus lanatus*	Gravel sludge, red mud and limestone	Phytostabilisation	Cd, Zn, Pb, As, Cu	4 years	Friesl-Hanl *et al.* (2009)
Dredged sediment from a section of canal located in Warrington, NW England	*S. atrocinerea*, *S. caprea* x *cinerea* x *viminalis* (Calodendron) and *S. viminalis* (Jorunn), *P. deltoides* x *nigra* (Ghoy) and *P. trichocarpa* (Trichobel), *A. cordata* and *A. glutinosa*		Phytoextraction / Phytostabilisation	Zn Pb Cu Cr Cd Ni As	8 years	Hartley *et al.* (2011)
Arable land irrigated with wastewater discharged from a smelter (Gansu Province, China)	*P. alba* var. *pyramidalis* Bunge		Phytoextraction / Phytostabilisation	Cd-, Cu-, Pb-, and Zn-	4 years	Hu *et al.* (2014)

(Continued)

Site description	Plant species	Soil management	Type of remediation technique	Pollutants	Experiment duration	References
Former maize field close to a Zn smelter (Lommel, Belgium)	168 willow clones (8 commercial and 160 experimental) and 105 poplar clones (5 commercial and 100 experimental)		Phytoextraction	Cd, Zn, Pb	3 years	Janssen *et al.* (2015)
Sediments from a semi-drained, derelict section of a canal (Warrington, NW England)	*A. cordata, A. glutinosa, A. incana, P. deltoides* x *nigra, P. trichocarpa, S. atrocinerea, S. carprea* x *cinerea* x *viminalis, S. fragilis , S. viminalis, S. viminalis* x *burjatica, S. viminalis* x *caprea, S. viminalis* x *schwerinni*	Manual and chemical weed control	Phytoextraction / Phytostabilisation	As, Cd, Cr, Cu, Pb, Zn organic pollutants	3 years	King *et al.* (2006)
Cd contaminated agricultural land (E Sweden)	*S. viminalis*	Fertilisation with N-fertilisers and/or sewage sludge	Phytoextraction	Cd	3 years	Klang-Westin and Eriksson (2003)
Cu-contaminated former wood preservation site (Gironde, SW France)	*Helianthus annuus*	Fertilisation (NPKS), irrigation, amendments (compost, dolomitic limestone and iron grit)	Phytostabilisation	Cu and polycyclic aromatic hydrocarbons	1 year	Kolbas *et al.* (2011)
Former waste disposal site (Antwerp, Belgium)	SRC with 13 poplar clones	Irrigation, mechanical and chemical (glyphosate) weed control	Phytoextraction	Zn, Al, Cu, Cd, Ni, Pb, Cr, Co, As	7 years	Laureysens *et al*, (2004)
Former sediment deposition during winter inundation (Merelbeke (Belgium)) and a dredged sediment soil (Deinze (Belgium))	*P. trichocarpa* x *P. deltoides* cv 'Beaupré'.		Phytoextraction	Cd, Zn, Mn and Cu	6 years / 10 years	Lettens *et al.* (2011)
Dredged sediment disposal site (Belgium)	*S. viminalis* ''Orm''		Phytoextraction	Cd, Cr, Cu, Ni, Pb, and Zn	1 growing season	Meers *et al.* (2005)

Site description	Plant species	Soil management	Type of remediation technique	Pollutants	Experiment duration	References
Dredged sediment disposal site (Menen, Belgium)	*S. fragilis* (clone 'Belgisch Rood'), *S. triandra* (Noir de Villaines)		Phytoextraction	Cd, Cr, Cu, Ni, Pb, and Zn	6 years	Mertens *et al.* (2006)
Polluted sites by deposition dust of a Zn/Pb smelter (Noyelles-Godault, France)	*Acer campestre*, *Acer pseudoplatanus*, *A. glutinosa*, *B. pendula*, *Fraxinus excelsior*, *Prunus avium*, *Quercus robur*, *Q. rubra*, *Robinia pseudoacacia*, *S. alba*, *S. caprea*, *S. purpurea* and the following hybrid poplars: *P. deltoides* × *nigra*, *P. tremula* × *alba*, *P. tremula* × *tremuloides*, *P. trichocarpa* × *deltoides*.		Phytoextraction / Phytostabilisation	Cd, Zn and Pb	12 years	Migeon *et al.* (2009)
Application of irrigation based on sewage sludge and final effluent waste water irrigation (Berkshire, England)	*P. trichocarpa* x *deltoides* 'Beauprè' and *P. trichocarpa* 'Trichobel',	Herbicide for weed control		Zn, Cu, Ni, Cd, Pb, Cr, Hg	3 years	Moffat *et al.* (2001)
Waste from smelting processes (Silesia, Poland)	Grass mixtures	Biosolids and lime application	Phytostabilisation	Zn, Cd and Pb	1 year	Stuczynski *et al.* (2007)
Former land-fill (Neuchâtel, Switzerland) capped with a top layer of contaminated compost originating from contaminated sewage sludge and uncontaminated soil and gravel.	*A. incana*, *B. pendula*, *Fraxinus excelsior*, *S. viminalis* and *Sorbus mougeotii*		Phytostabilisation / Phytoextraction	Cd, Zn, Cu	3 years	Rosselli *et al.* (2003)
Former maize field close to a metal smelter (Lommel, Belgium)	SRC (seven commercial willow and poplar clones)	Weed control mechanically and glyphosate based herbicide	Phytoextraction	Zn, Cd, Pb and Cu	2 years	Ruttens *et al.* (2011)

(Continued)

Site description	Plant species	Soil management	Type of remediation technique	Pollutants	Experiment duration	References
Metal(loid)-contaminated wastes derived from pyrite ore roasting for sulphur extraction were discharged adjacent to a chemical plant at Torviscosa (Italy)	*P. alba*, *P. nigra*, *P. tremula* and *S. alba*	Fertilised with NPK and irrigation	Phytostabilisation	As, Co, Cu, Pb and Zn	6 months	Vamerali *et al.* (2009)
Diffusely contaminated region by smelter activities (Campine region in Flanders, Belgium)	*S. alba* var. Alba ('Belders'), *S.* x *rubens* var. basfordiana ('Belgisch Rood'), *S. viminalis ('Christina')*, *S. triandra* x *viminalis* ('Inger'), *S. viminalis* ('Jorr'), *S. dasyclados* ('Loden'), *S. schwerinii* x *viminalis* ('Tora') and *S. triandra* ('Zwarte Driebast')	Limed one month before planting and mechanical weeding	Phytoextraction	Pb, Cd, Zn	1 growing season	Van Slycken *et al.* (2013)
Dredged sediment disposal sites in Menen (Belgium)	*S. viminalis* 'Orm'		Phytoextraction	Cd, Pb, Zn, Cu	1.5 year	Vervaeke *et al.* (2003)
Cd, Pb and Zn-contaminated agricultural soil (Podlesí, Czech Republic)	([*S. schwerinii* × *viminalis*] × *viminalis*); *S.* × *smithiana*; *P. aximowiczii* × *nigra*; *P. nigra* clone Wolterson		Phytoextraction	As, Cd, Pb and Zn.	4 years	Zárubová *et al.* (2015)

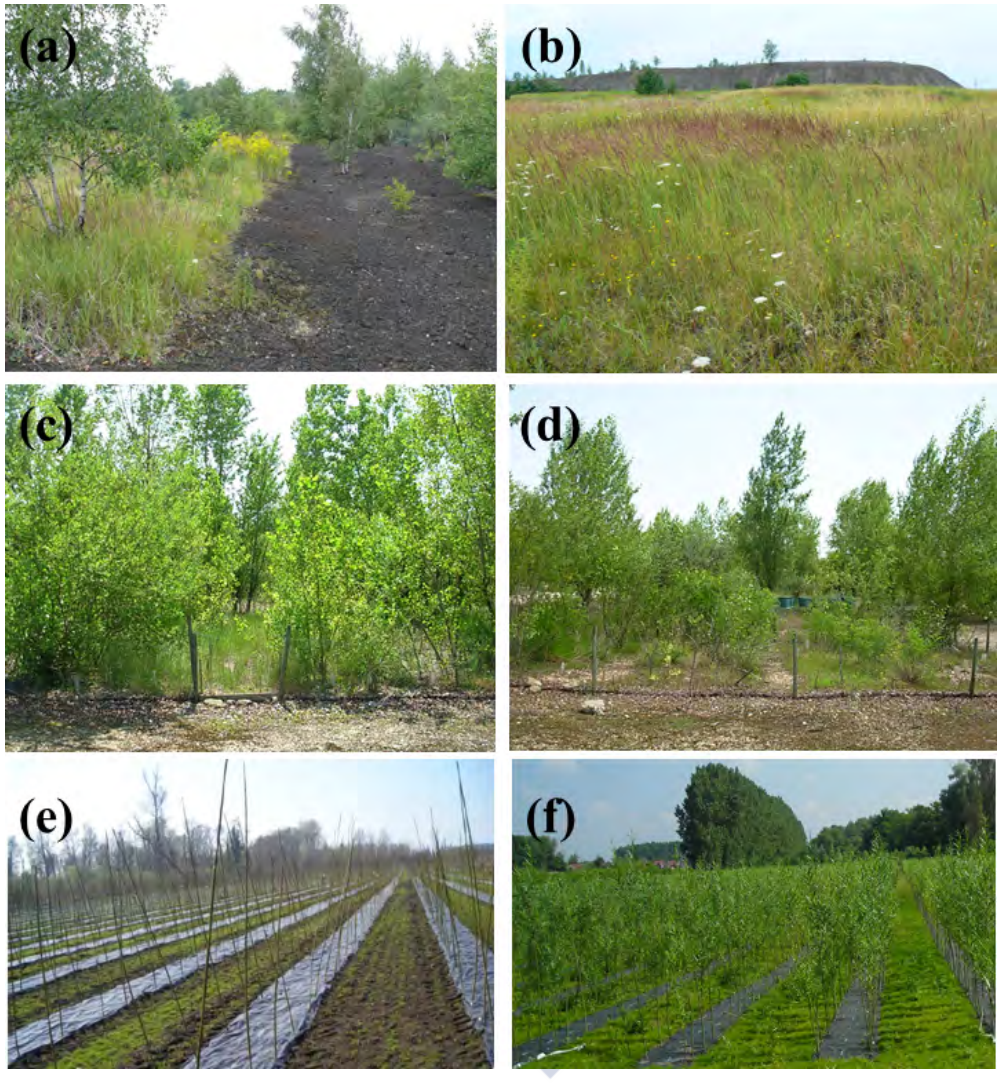


Fig 1.7. Field–scale phytostabilisation trials in (a-b) the Piekary site in Poland where smelter-affected soils were amended with biosolids and lime and planted with a grass cover, (c-d) Biogeco site in France planted with ectomycorrhizal poplar and willows on the left and unmycorrhizal poplar and willows on the right and (e-f) Phytosed Ec site in France where soils were amended with Thomas Basic Slag and planted with *Deschampsia cespitosa (*vegetation cover) and *Salix* SRC

The practice of liming (chalk or limestone ($CaCO_3$), quicklime (CaO), or hydrated lime ($Ca(OH)_2$) to increase soil pH has been commonly used and associated effects on metal(loid) mobility are well known (reviewed by Vangronsveld *et al.*, 2009). Liming can also be ineffective in metal(loid) immobilisation but this is usually metal(loid)-dependant. McLaughlin *et al.* (2000) reported an increase in Cd accumulation in potato tubers after lime addition. Addition of lime can also potentially mobilise metalloids such as As (Jones *et al.*, 1997). The long-term effects and sustainability of liming are also questionable (Gray *et al.*, 2006; Ruttens *et al.*, 2010). For this reason, other alkaline amendments, and different combinations of amendments, have been evaluated. Amendments rich in metal(loid) oxides combined with compost, fertilisers, beringite, cyclonic ashes or lime enhanced plant growth (Bes and Mench, 2008; Vangronsveld *et al.*, 2009). The combination of iron grit with lime and compost was more effective in reducing Cu concentrations in soil pore water than individual amendments (Bes and Mench, 2008). Application of cyclonic ashes (formerly known as beringite) which are rich in clay minerals induced a strong decrease in plant-available Pb, Zn and Cd concentrations, and restored vegetation, in the Zn-smelter affected area of Lommel (Belgium). Moreover, this effect was still observed after 12 years (Vangronsveld *et al.*, 2000a; Vangronsveld *et al.*, 2000b). The application of red muds (an alkaline and Fe/Al oxide-rich by-product of the aluminium industry) led to a decrease of up to 63%, 425% and 50% in soil Zn, Cd and Ni extractability in a 15-month pot study. However, similar effects of these amendments were not observed when applied at a field scale (Friesl-Hanl *et al.*, 2006). Many of these amendments are by-products of industrial activities and are therefore inexpensive and available in large amounts (Mench *et al.*, 1998). Moreover, their use can provide an environmentally sustainable means for the recycling of such residues (instead of incineration).

Organic matter addition can improve soil physical properties, water infiltration and WHC, provide micro- and macro-nutrients for plant growth and stimulate microbial activity. In addition, the formation of stable organo-metal(loid) complexes can reduce the availability of metal(loid)s to plants for uptake (Clemente and Bernal, 2006). Authors have recommended the use of organic amendments with a low mineralisation rate within a neutral pH range in order to avoid the release of metal(loid)s and reduce their uptake and bioaccumulation by plants (Blake and Goulding, 2002). Although the most widely described effect of OM additions on metal(loid) mobility is one of immobilisation, some studies have

also shown an enhanced metal(loid) solubilisation (Almås *et al.*, 1999; Clemente and Bernal, 2006; Tandy *et al.*, 2009). The effects of organic amendments on metal(loid) availability depend on the nature of the OM, and on the particular soil type and elements concerned (Clemente *et al.*, 2005; Ruttens *et al.*, 2006a; Goecke *et al.*, 2011; Kumpiene *et al.*, 2011; Lagomarsino *et al.*, 2011). Other organic amendments, such as municipal wastewater, sewage sludge, landfill leachate or wood ash, have also been applied in GRO (Marchiol *et al.*, 2007; Adler *et al.*, 2008). A key element in the sustainability and (self)-maintenance of phytostabilisation techniques is the monitoring of amendment-induced effects and the need for new applications (especially in the case of liming or OM addition), since soil re-acidification after liming or OM mineralisation may result in the re-mobilisation of sorbed metal(loid)s (Tisch *et al.*, 2000).

Nutrient deficiency is a major limiting factor for plant growth in TE-contaminated areas, and is especially limiting in environments such as mine tailings (Ye *et al.*, 2002). Fertilisation of tailings soil can facilitate plant survival, alleviate growth inhibition and ensure a better plant development. Pioneer studies already demonstrated the importance of fertilisation during the re-vegetation of TE -contaminated mining areas in the USA (Pancholy *et al.*, 1975; Cundell, 1977). Fertilisation regimes can be designed with the aim of improving plant growth and establishment but they can also be designed to increase plant uptake of TEs. Inorganic fertiliser application can affect TE bioavailability, and much research has been carried out towards a better management of fertilisers and reduction in food-chain transfer of TE in agriculture (McLaughlin *et al.*, 1996; Mench, 1998; McLaughlin *et al.*, 1999; McLaughlin and Singh, 1999; Singh *et al.*, 2011 Chaney, 2012). The type and amount of fertiliser used and interactions between TE and major nutrients (N, P and S) and among TE themselves (e.g. Zn–Cd and Fe–Cd) are key-players in TE uptake by crops (Tiller *et al.*, 1997; Mench, 1998). Plant availability of Cd can be affected directly through the addition of Cd as a contaminant in P fertiliser, or indirectly through ion exchange reactions in the soil solution (McLaughlin and Singh, 1999; Wångstrand *et al.*, 2007). Furthermore, fertilisers can influence Cd speciation and complexation. Chloride can increase crop Cd uptake more than other soil factors due to the formation of chloride complexes (Smolders *et al.*, 1998), and this is particularly pronounced in alkaline soils (Hattori *et al.*, 2006). Ammonium fertilisers frequently cause higher Cd concentrations in crops than nitrate fertilisers as a result of the pH decrease caused by nitrification or plant uptake of NH_4^+ (Grant *et al.*, 1999). The chemical form of

fertilisers can therefore be selected according to the GRO technique to be implemented: in the case of phytoextraction, ammonium-, sulfate- or chloride-based fertilisers may be managed so as to enhance the phytoavailability and hence uptake of metallic cations, while in the case of GRO where TE stabilisation is the aim avoiding certain fertiliser types can minimise plant TE uptake. Ammonium-sulphate enhanced metal uptake and accumulation (Cd, Zn and Pb) by tobacco mutants in phytoextraction field trials, and this treatment was shown to be more efficient than the addition of chelating agents such as citric acid or nitrilotriacetic acid (NTA) (Herzig *et al.*, 2005). The application of elemental S may increase the metal(loid) solubility for plant uptake (Wenger *et al.*, 2002; Wang *et al.*, 2008; Iqbal *et al.*, 2012). Microbial oxidisation of elemental S leads to the production of sulphuric acid and consequent decrease of soil pH (depending on the soil pH buffering capacity). Sulphur oxidation along with microbial and root respiration might also lead to partly anoxic conditions in the rhizosphere, inducing reductive dissolution of manganese oxides, which further enhances the Zn and Cd solubility (Iqbal *et al.*, 2012). However, a site-specific balance between increasing metal (loid) solubility and potential negative effects due to decreasing pH needs to be found. A decrease in soil pH to values below 4.5-5.0 would lead to irreversible soil degradation and massive leaching of macronutrients such as Ca, Mg and K and to an increase in potential Al phytotoxicity (Fässler *et al.*, 2012). Fässler *et al.* (2012) increased soluble Cd and Zn after elemental S application by decreasing soil pH. Hoefer *et al.* (2015) reported an increase in the Cd and Zn availability of a calcareous soil after S^0 addition and at the same time an increase in shoot metal concentration by willows was found. These authors attributed the effects to soil acidification after sulphur application but also to the co-solubilisation of metals presents in Mn-rich minerals. Improvements in the biomass production of hyper-accumulators using inorganic fertilisers have been also reported (Robinson *et al.*, 1997; Schwartz *et al.*, 2003; Bani *et al.*, 2007).

Plant cropping patterns to improve gentle remediation options performance

Crops patterns can be designed so as to improve plant biomass and nutrition, and/or enhance or mitigate TE availability and uptake and accumulation (Kidd *et al.*, 2015). Most plant species selected as relevant candidates for GRO have been studied as monocultures. Monocultures can lead to a decline in biomass yields due to the depletion of nutrients, occurrence of diseases, pests, and weeds, and have a negative effect on soil fertility (Facknath and Lalljee, 2000; Lasat,

2000; Mench *et al.*, 2010). Reductions in biomass yield can reduce the plants' phytoextraction capacity. While alternative cropping patterns, such as rotations or intercropping, can improve plant productivity and nutrition, enhance biodiversity and habitat, or aid pest control. For both improved sunflowers and tobacco mutant lines a crop rotation scheme is obligatory to avoid plant diseases over a longer period of time (Herzig *et al.*, 2014). Crop rotation with non-hosts, such as rapeseed, corn, and winter fodder pea is suggested. Although a minimum rotation period of four years is recommended between successive sunflower crops, good results have been obtained with three year crop rotations of sunflower – tobacco – corn, or a two year rotation of sunflower – tobacco followed by winter fodder pea in Zn-contaminated agricultural soils which were polluted with deposits of a former hot dip Zn factory (Bettwiesen, Switzerland; Figure 1.8) (Herzig *et al.*, 2005; Nehnevajova *et al.*, 2009; Herzig *et al.*, 2014). Winter fodder pea (green fertiliser) can still grow during winter and avoids periods of bare soil, thus reducing the risks of transfer of the contaminated soil material by wind or water erosion processes and the leaching of TE's during this time. After five years of the sunflower-tobacco rotation scheme, bioavailable (labile) Zn concentrations were reduced by up to 70% in top soils (Herzig *et al.*, 2014). Sunflower-tobacco rotations and tobacco–sunflower–vetiver rotation were also successfully implemented in Cu-contaminated soils at a former wood preservation site (Kolbas, 2012). As discussed above, SRC of fast-growing tree species (such as *Salix* and *Populus* spp.) is a common technique for production of biomass for conversion to energy or as a feedstock for other chemical processes (Landberg and Greger, 1994; Riddell-Black *et al.*, 1997; Kayser *et al.*, 2000; Hammer *et al.*, 2003) and has been successfully implemented within GRO on metal(loid) contaminated soils (Dimitriou *et al.*, 2012). These woody species show the ability to re-sprout from the stumps after harvests which are performed at short time intervals (i.e. 2 – 6 years) (Dimitriou *et al.*, 2012). Management practices for SRC e.g. weed control, planting, fertilisation, and harvesting, are in between agriculture and forestry and the equipment used resembles more that of common agriculture, although they are specially designed for SRC. Harvest of SRC is generally carried out in winter or early spring (Dimitriou *et al.*, 2012). The use of polyclonal stands of *Salix* and *Populus* clones rather than monocultures has also been recommended when implementing SRC in phytostabilisation options as a means of reducing disease incidence, particularly from *Melampsora rust* (McCracken and Dawson, 2003; French *et al.*, 2006).

Intercropping of plants is often used in agriculture and aims to stimulate interspecific below-ground interactions, which may result in improved nutrient availability and increased yield of crops (Wieshammer *et al.*, 2007). Nutrient deficiency is a common characteristic of contaminated soils, but intercropping with leguminous plants can supply nutrients (through N_2 fixation, transfer of fixed N and mobilisation of P due to rhizosphere acidification) to phytoremediating crops. Intercropping can also alter conditions in the shared rhizosphere and thereby affect the availability of selected TE to neighbouring plants (Tang *et al.*, 2012). For phytoextraction purposes, combining hyperaccumulators (with relatively restricted root proliferation) with deep-rooting species (such as willows) could favour a more complete and deeper use of soil resources, or even target deeper contaminants (Keller and Hammer, 2005). Combining low-metal(loid) accumulating crops with hyperaccumulators or other appropriate metal(loid)-accumulating plants could permit simultaneous remediation with agricultural production on TE-contaminated soils (Tang *et al.*, 2012). Co-cropping of the hyperaccumulator *N. caerulescens* with a non-accumulator *Thlaspi arvense* increased the growth of the non-accumulator and reduced its Zn uptake, while at the same time Zn uptake was enhanced in the hyperaccumulator (Whiting *et al.*, 2001b). The co-cropping of metal(loid) hyperaccumulators with edible crops, such as maize, soybean or ryegrass, has also been shown to significantly improve the phytoextraction capacity of Zn/Cd hyperaccumulators (Jiang *et al.*, 2009; Jiang *et al.*, 2010). Moreover, multi-cropping of various hyperaccumulators resulted in a higher total biomass production and metal(loid) accumulation (Lucisine *et al.*, 2014; Rue *et al.*, 2015). Plant diversity and composition induces a wider variety of released rhizodeposits (Zak *et al.*, 2003; Benizri and Amiaud, 2005) thus generating an increase in soil bacterial community diversity and improvements in soil functionality (Benizri and Amiaud, 2005; Gao *et al.*, 2010). Intercropping of agricultural crops with legumes has nutritional advantages compared to monocultures due to the ability of legumes to establish symbiotic interactions with N_2 fixing bacteria (Bloem *et al.*, 2009; Liu *et al.*, 2013). Legumes include economically important grain crops, oilseed crops, forage crops, and agroforestry species. Maize has traditionally been intercropped with legumes within conservation agriculture to inhibit soil degradation and control pests (Sekamatte *et al.*, 2003; Ngwira *et al.*, 2012). More recently, the legume-rhizobia symbiosis has been applied in metal(loid)-contaminated soils (Carrasco *et al.*, 2005; Dary *et al.*, 2010; Hao *et al.*, 2013). Native leguminous species such as *Medicago*, *Trifolium*, *Vicia*, *Lotus* or *Lupinus* can be found growing in

metal(loid)-contaminated sites (Prasad and De Oliveira Freitas, 2003; Carrasco *et al.*, 2005; Hao *et al.*, 2013). Cluster roots of species such as *Lupinus* are also known to be involved in inorganic P solubilisation by organic acids exudation in response to P deficiency (Keerthisinghe *et al.*, 1998; Neumann and Martinoia, 2002; Vance *et al.*, 2003). Woody trees such as alders (*Alnus* sp.) also form symbioses with N_2-fixing microorganisms (such as the genera *Frankia* sp.), ectomycorrhizae and arbuscular mycorrhizal fungi (Tarrant and Miller, 1963; Taleshi *et al.*, 2009). Keleberda (1978) studied spoil heaps of a Mn quarry in Ukraine planted with Scotch pine (*Pinus sylvestris*) and black alder (*Alnus glutinosa*). Tree growth was healthier in mixed rather than in monoculture stands, and the presence of alder much improved the soil fertility. Nitrogen fixation can decrease soil pH due to nitric acid accumulation in the rhizosphere (Van Miegroet and Cole, 1984), which for phytoextraction purposes can in turn induce an increase in TE bioavailability to the co-cropped TE-accumulators.

The role of plant associated-bacteria in gentle remediation options effectiveness

Plant establishment and growth in TE-contaminated soils can be improved using plant growth-promoting bacteria (PGPB) and fungi (including mycorrhizae and endophytic fungi). This introduction will only discuss the beneficial effects of plant-associated bacteria on phytoremediation processes in TE-contaminated soils, however similar interactions also occur in organic-contaminated sites (see reviews by Vangronsveld *et al.*, 2009; Álvarez *et al.*, 2012; Khan *et al.*, 2013; Segura and Ramos, 2013) as well as between plants and their associated fungi (see reviews by Göhre and Paszkowski, 2006; Lebeau *et al.*, 2008). Plant-associated bacteria include several groups: endophytic bacteria, which colonise the internal tissues of plants without causing negative effects on plant health; phyllospheric bacteria, which inhabit the external surfaces of plant parts; and rhizobacteria, which are present in the rhizosphere soil in direct contact with plant roots (Sessitsch and Puschenreiter, 2008; Weyens *et al.*, 2009). Using soil bacteria as bioferilisers is a common approach in agriculture to avoid excessive input of fertilisers (which can be a further source of pollution). The use of bacteria, which are able to fix atmospheric N_2 or to solubilise unavailable P and Fe, have been identified as good candidates for sustainable biomass production in TE-contaminated lands (Weyens *et al.*, 2009). Higher plants present associations with diazotrophic bacteria which can reduce N_2 to the NH_4^+ form (that is then available to plants for uptake), occurring in the form of nodules (symbionts) or as free living cells. Inorganic P-solubilising

bacteria are able to mineralise insoluble phosphate and make it accessible for plant uptake (Rodriguez and Fraga, 1999; Vessey, 2003). Siderophore compounds are Fe(III)-specific chelating agents, these compounds are produced by bacteria and can bind insoluble Fe(III) making it available for plants (Crowley and Kraemer, 2007). Bacteria can also produce phytohormones such as auxins, cytokinins and gibberellins, which stimulate plant growth and development (Tanimoto, 2005; Taghavi *et al.*, 2009). Phytohormones are involved in root growth and root hair proliferation, the stimulation of cell division and tissue expansion, and in stomatal opening or modifying plant morphology and tissue extension (Hare *et al.*, 1997; Vessey, 2003; Taghavi *et al.*, 2009). Plant associated-bacteria can also reduce plant stress through the synthesis of ACC deaminase which reduces the high levels of ethylene by consuming its immediate precursor, the 1-aminocyclopropane-1-carboxylic acid (ACC) (Glick, 2014). Other well-known mechanisms of PGPBs include increasing water uptake, alteration of root morphology, production of antibiotics and the induction of plant defence mechanisms (Lin *et al.*, 1983; van Loon and Bakker, 2003; Kidd *et al.*, 2009). Plant associated-bacteria can also influence TE mobility, and consequently plant uptake and accumulation (see reviews by Gadd, 2004; Haferburg and Kothe, 2007; Weyens *et al.*, 2009; Sessitsch *et al.*, 2013). Plant-microbial interactions greatly influence TE mobility and availability to plants for uptake (Anderson *et al.*, 1993; Marschner *et al.*, 2004). Three specific mechanisms of microbial-induced mobilisation of TE and increased plant metal(loid) accumulation are: (i) increasing root surface area and hair production, (ii) increasing element solubility (iii) increasing soluble element transfer from the rhizosphere to the plant (Whiting *et al.*, 2001a). An increase in soil metal(loid) mobility and availability can result in increased metal(loid) uptake and therefore phytoextracted metal(loid) (Kidd *et al.*, 2009; Weyens *et al.*, 2009; Sessitsch *et al.*, 2013). In contrast, microorganisms can immobilise metal(loid)s through sorption to cell components or exopolymers, transport and intracellular sequestration, release of metal(loid) binding compounds or precipitation as insoluble organic or inorganic molecules (Gadd, 2004). Mineralisation of dissolved metal(loid)-organic complexes may be another cause of microbial mediated immobilisation (Gadd, 2004). In contrast, precipitated metal(loid)s can be solubilised by acidification, chelation and ligand-induced dissolution (Gadd, 2004). Oxalate, malate and citrate are some of the most important organic acids identified in root and microbial exudates (Ehrlich, 1998; Jones, 1998).

The last two decades has seen an increase in the search for candidate bacte-

rial strains with potential application in GRO. Natural metal(loid)-enriched areas (such as serpentine soils) or TE-contaminated sites are not only a source of interesting plant species for application in phytoremediation but also of microorganisms (Schippers *et al.*, 1995; Batty, 2005; Mengoni *et al.*, 2010). Numerous studies have focused on the isolation and characterisation of cultivable microorganisms from this type of substrate (Lodewyckx *et al.*, 2002; Abou-Shanab *et al.*, 2003; Idris *et al.*, 2004; Pal *et al.*, 2005; Barzanti *et al.*, 2007; Grandlic *et al.*, 2008; Weyens *et al.*, 2009).

To be useful in phytoremediation processes the selected bacterial inoculants must be able to tolerate the potentially toxic concentrations of metal(loid)s present in the substrate but should also be adapted to the specific conditions of the site (such as nutrient deficiency). In serpentine areas, these abiotic factors have often been suggested to be the cause of the low number of microorganisms in these soils (Lipman, 1926; Acea and Carballas, 1986; Pal *et al.*, 2005). Nonetheless, bacterial communities in metal(loid)-enriched environments present a high genetic diversity. Moreover, highly metal(loid)-tolerant bacterial strains have been isolated from serpentine soils (Mengoni *et al.*, 2001; Abou-Shanab *et al.*, 2003; Amir and Pineau, 2003; Pal *et al.*, 2005). TE-contaminated mine tailings are also an important source of highly metal(loid)-tolerant bacteria. Numerous metal(loid)-tolerant bacterial strains belonging to diverse taxonomic groups have been isolated from mining sites, as well as domestic and industrial wastes (Stoppel and Schlegel, 1995; Schippers *et al.*, 1995; Batty, 2005; Sprocati *et al.*, 2006).

Bacterial densities are higher in the rhizosphere than in non-vegetated soil, due to the release of easily degradable labile C compounds (Rouatt, 1959; Grayston *et al.*, 1998). The rhizosphere of (pseudo)metallophyte plants provides a metal(loid)-rich niche where populations of metal(loid)-resistant bacteria can establish (Mengoni *et al.*, 2001; Lodewyckx *et al.*, 2002; Abou-Shanab *et al.*, 2003; Aboudrar *et al.*, 2007; Kuffner *et al.*, 2008; Becerra-Castro *et al.*, 2009; Janssen *et al.*, 2015). Schlegel *et al.* (1991) detected higher numbers of TE-resistant bacteria in the rhizosphere of Ni accumulating plants. Similarly, Idris *et al.* (2004) identified a high number of different Ni-resistant bacteria in the rhizosphere of the Ni-hyperaccumulator *Noccaea goesingensis*. Cultivation of bacteria on Ni-containing medium resulted mostly in the isolation of *Methylobacterium* spp., an alphaproteobacterial genus, as well as *Rhodococcus* spp. and *Okibacterium* spp., belonging to the *Actinobacteria* (or Gram-positive

bacteria with a high G+C content) (Idris *et al.*, 2004). Mengoni *et al.* (2001) isolated Ni-resistant rhizobacterial strains, mostly belonging to the genera *Pseudomonas* sp. Rhizosphere bacteria associated with pseudometallophytes growing in a Pb/Zn mining area were primarily affiliated with the phylum *Actinobacteria*. At the genus level, the majority belonged to the genera *Streptomyces* (61%), *Tsukamurella* (18%) or *Pseudomonas* (18%). Kuffner *et al.* (2010) isolated and characterised Zn-resistant rhizosphere bacteria of Zn-accumulating willows (*Salix caprea*) and found a high number of *Bradyrhizobia*, various *Betaproteobacteria*, *Actinobacteria* and members of the *Bacteroidetes*/ *Chlorobi*. The discovery of new bacterial strains may contribute towards further developing the potential application of bacterial inoculants in phyto-extraction or –stabilisation technologies (Ortíz-Castro *et al.*, 2009; Haslmayr *et al.*, 2014).

On the basis of these studies, numerous bacterial isolates (rhizosphere or endophytic bacteria) associated with metal(loid)-tolerant or metal(loid)-(hyper) accumulating plants have been identified as potentially useful inoculants within phytoremediation systems. Most studies have evaluated the effects of re-inoculating host plants with their associated isolated strains (Abou-Shanab *et al.*, 2003; Abou-Shanab *et al.*, 2006; Li *et al.*, 2007; Ghosh *et al.*, 2011; Cabello-Conejo *et al.*, 2014a). However, the specificity of these plant-bacterial combinations is unclear, and some inoculants have been shown to have beneficial effects on a wide range of plant hosts (Grandlic *et al.*, 2008; Ma *et al.*, 2011; Becerra-Castro *et al.*, 2012). Most strains were identified and tested for phytoextraction purposes, in this technique a simple improvement in biomass can result in an increase in the overall TE removal (Sessitsch *et al.*, 2013). In a meta-analysis carried out by (Sessitsch *et al.*, 2013) including more than 70 publications and 738 individual cases the authors found that bacterial inoculation was generally more successful in promoting plant growth and biomass production (60% of the total analysed cases) than influencing shoot metal(loid) accumulation (only 30% of the cases showed a significant increase in shoot TE concentration, and 16% a decrease in TE concentration).

Enhanced plant growth and phytoextraction efficiency has been observed in several hyperaccumulating plants after inoculation with either rhizosphere or endophytic bacterial strains, including *A. serpyllifolium* (Cabello-Conejo *et al.*, 2014b), *N. caerulescens* (Aboudrar *et al.*, 2013; Visioli *et al.*, 2015), *A. murale* (Abou-Shanab *et al.*, 2006; Durand *et al.*, 2016) or *Sedum alfredii* (Guo *et al.*, 2011). Similar bacterial-induced stimulation in growth has been found in high

biomass-producing plant species (both accumulator and excluder phenotypes) (Belimov *et al.*, 2004; Shilev *et al.*, 2006; Kuffner *et al.*, 2008; Mastretta *et al.*, 2009; Kuffner *et al.*, 2010; Li and Ramakrishna, 2011; Becerra-Castro *et al.*, 2012; Kolbas *et al.*, 2015). Zaidi *et al.* (2006) reported that a *Bacillus subtilis* strain was able to promote the growth of *Brassica juncea* and thereby increased Ni removal from a spiked-contaminated soil. The strain was shown to produce IAA, which might have been responsible for the promotion in plant growth. Similarly, Dell'Amico *et al.* (2008) observed IAA-, ACC deaminase- and siderophore-producing rhizosphere isolates promoted growth of *Brassica napus* in presence of Cd. Kuffner *et al.* (2008) revealed a positive effect of rhizosphere bacteria on accumulated TE contents in willows (*Salix caprea*). Microbial inoculants increased Cd and Zn translocation factors from roots to leaves of *S. caprea* grown in a moderately contaminated soil (De Maria *et al.* 2011). Babu *et al.* (2015) found that inoculation with the root endophyte (*Pseudomonas koereensis* AGB-1) increased plant growth of *Miscanthus sinensis* growing in mine tailings at the same time as an increase in plant metal(loid) uptake. In the same study a decrease in antioxidant enzyme activities (catalase or superoxide dismutase) which are induced under metal(loid) stress was also found. Extracellular sequestration of metal(loid)s by this bacterial strain was revealed using Transmission Electron Microscopy (TEM)-micrographs, which were attributed to the formation of complexes between metal(loid)s and exopolymers produced by the strain to reduce metal(loid) toxicity. Adediran *et al.* (2015) found that *Rhizobium leguminosarum* promoted *Brassica juncea* growth in the presence of toxic concentrations of Zn, and this effect was observed when the strain was present alone or in combination with *Pseudomonas brassicacearum.* The improved growth and increased meal accumulation observed in the inoculated plants was attributed to the storage of Zn in the form of Zn-phytate and Zn-cysteine in the roots. No improvement in growth was observed in the absence of metal, suggesting that bacteria-induced metal chelation is a key mechanism of plant growth promoting bacteria in toxicity attenuation. Bacterial-induced promotion in plant growth has also been documented in phytostabilisation-orientated trials. Petrisor *et al.* (2004) found that co-inoculating the N_2-fixing *Azotobacter chroococcum* with the P-solubiliser *Bacillus megaterium* led to a better establishment and growth of indigenous herbaceous plants in phophogypsum mine tailings compared to inorganic fertilisation. In this study, bacterial inoculation also decreased the uptake of Cd, Zn and Mn by plants. Salt *et al.* (1999a) also reported a decrease in root Cd uptake of Indian

mustard (*Brassica juncea*) due to Cd precipitation at the roots, which was promoted by the inoculated rhizobacterial strains. Inoculation of *Lupinus luteus* with *Bradyrhizobium* sp. increased both plant biomass and nitrogen content in a field experiment in the Aznalcóllar mine (the area was affected by a toxic spill). Co-inoculation of lupines with a consortium of metal(loid) resistant PGPR (including *Bradyrhizobium* sp., *Pseudomonas* sp. and *Ochrobactrum cytisi*) produced an additional improvement in plant biomass. At the same time, decreases in both shoot and root metal(loid) accumulation were observed, which could be due to a protective effect exerted on the plant rhizosphere (Dary *et al.*, 2010). Touceda-González *et al.* (2015) also found promising results using bacterial inoculants in combination with immobiliser amendments to reduce the negative effects of Cd/Zn on growth and metal accumulation in *Zea mays*, as well as on soil microbial communities. Cases of bacterial-induced changes in soil metal(loid) mobility can also be found in the literature. Microbial activity was associated with an enhanced release of Co and Ni in ultramafic soils of New Caledonia (Amir and Pineau, 2003). Numerous authors have shown bacterial-induced TE mobilisation or solubilisation of TE-rich minerals using *in vitro* testing. This mobilisation has frequently been attributed to the action of bacterial siderophores or to the release of organic acids (Lodewyckx *et al.*, 2002; Abou-Shanab *et al.*, 2003; Braud *et al.*, 2009). Cell-free culture filtrates of rhizobacterial strains isolated from hyperaccumulating subspecies of *A. serpyllifolium* increased Ni extraction from ultramafic soils (Becerra-Castro *et al.*, 2011). TE-mobilising metabolites of various *Actinobacteria* mobilised soil Zn and /or Cd (Kuffner *et al.*, 2010). No clear connection between the phenotypic traits of isolates and their ability to mobilise soil TE was found. For example, strains which were not able to produce siderophores were found to strongly mobilise soil TE, and this was most likely due to the production of other secondary metabolites (Kuffner *et al.*, 2008; Kuffner *et al.*, 2010). In addition, in some cases siderophore-producing bacteria have led to a decrease in the bioavailability of Zn and Cd (Kuffner *et al.*, 2008; Kuffner *et al.*, 2010) or Ni (Becerra-Castro *et al.*, 2011). It has been suggested that bacterial-secreted exopolymers may complex TE leading to a reduced availability for plants (Diels *et al.*, 1995; Kunito *et al.*, 2001). TE mobilisation by rhizosphere bacteria and simultaneous promotion of uptake has been observed in annual crops. Cd- and Pb-mobilising strains enhanced the uptake of TE in tomato (Jiang *et al.*, 2008), while Ni-solubilising strains increased Ni uptake by different subsp. of *Brassica* (Ma *et al.*, 2009). Abou-Shanab *et al.* (2006) found increases in the leaf Ni concentration of *A. murale* after inoculation with *Microbacterium oxydans*

AY509223 which under *in vitro* conditions was able to produce organic acids and siderophores. Guo *et al.* (2011) found increases in Cd, Pb and Zn uptake by the hyperaccumulator *S. alfredii* by siderophore-producing and P-solubilising microorganisms. Finally, Becerra-Castro *et al.* (2013) observed increased Ni uptake and accumulation by *A. serpyllifolium* after inoculation with Ni-solubilising rhizobacterial strains.

Comparisons between inoculation studies can be hindered due to the fact that the experimental conditions and inoculation method differ widely. The host plant species, soil type and properties, and nature and type of contamination (mono - or poly-metallic contamination, co-contamination with organic and inorganic pollutants, pollutant concentration, anthropogenic, geogenic, spiked contamination), are important factors determining whether or not a beneficial bacterial-induced effect is observed (Weyens *et al.*, 2010; Becerra-Castro *et al.*, 2012; Sessitsch *et al.*, 2013; Cabello-Conejo *et al.*, 2014a). Moreover, inoculation methods differ greatly between studies (inoculation of seed/plant/soil, number/timing of inoculation events, bacterial cellular densities) and these aspects are likely to influence the final effects of such plant-microbial associations. Table 1.4 summarises the different experimental conditions and inoculation methods used in some recent phytoextraction-orientated inoculation studies. The most common methods are seed and soil inoculation, while plant inoculation and experiments including more than one inoculation event are rare. Bacterial densities of the inoculum generally fluctuate between 10^7- 10^8 Colony forming units (CFUs) mL^{-1}, however the total density of inoculated bacteria per pot (CFUs per pot) is highly variable due to the different amounts of soil used. According to Malusá *et al.* (2012), the commercial use of microbial inoculants has been limited due to the inconsistency of the results obtained. There is therefore a need to test the effects of different inoculation methods on plant growth promotion and metal(loid) removal under standardised conditions. Additionally, the success of bacterial inoculants may be limited by a low proliferation and survival in the inoculated medium. Plants and microbes compete for resources, including nutrients and water, and in soils with low nutrient availability (as is commonly the case at contaminated sites) resource competition may become a limiting factor of bacterial growth and establishment (Kaye and Hart, 1997; Moorhead *et al.*, 1998; Joner *et al.*, 2006; Unterbrunner *et al.*, 2007). Addition of soil amendments to overcome this competition for nutrients may be required to ensure bacterial survival. Moreover, the performance of these bacterial inoculants under natural conditions will have to be investigated in detail.

Table 1.4. Summary of bacterial inoculation methods used in phytoextraction-orientated studies.

Bacterial inoculant	Plant Host	Soil / substrate type	Amount of soil / substrate	Experiment duration	Inoculation method			Re-inoculation (weeks)	Cellular density	Reference
					Plant	Seed	Substrate			
Mixed inoculum: *Bacillus pumilus* and *Micrococcus* sp	*Noccaea caerulescens*	Serpentine Ni-rich soil	100 g	32 d		X				Aboudrar *et al.* (2013)
Acidovorax avenae,Clavibacter xyli, Microbacterium sp., *Rhizobium galegae*	*Alyssum murale*	(1) low Ni soil (2) moderate Ni-serpentine soil (3) Ni-rich serpentine soil	500 g	60 d		X			20´ shaking in 7.4 x 10^8 CFUs mL^{-1}	Abou-Shanab *et al.* (2006)
14 strains isolated from native plants	*Festuca pratensis* *Salix viminalis*	Perlite:quartz sand mixture with Hoagland solution suplemented with Cd and Zn.		49 d			X		9 mL 1 x 10^7 CFUs mL^{-1}	Becerra-Castro *et al.* (2012)
15 strains isolated from rhizosphere of *A. serpyllifolium*	*Alyssum pintodasilvae*	Perlite:quartz sand mixture with Hoagland solution suplemented with Ni.		70 d			X		9 mL 1 x 10^8 CFUs mL^{-1}	Cabello-Conejo *et al.* (2014a)
Microbacterium sp., *Arthrobacter* sp., *Streptomyces* sp		(1) Serpentinitic region of Trás-os-Montes (2) Contaminated Ni soil		150 d					2 mL 1 x 10^8 CFUs mL^{-1}	

Bacterial inoculant	Plant Host	Soil / substrate type	Amount of soil / substrate	Experiment duration	Inoculation method			Re-inoculation (weeks)	Cellular density	Reference
					Plant	Seed	Substrate			
Pseudomonas sp, *Alcaligenes* sp, *Mycobacterium* sp	*Brassica napus*	Commercial soil artificially contaminated with $CdCl_2$	320 g	20 d		X			Incubated in 40 mL 1 x 10^8 CFUs mL^{-1}	Dell'Amico *et al.* (2008)
Streptomyces (AR17) and *Agromyces* (AR33)	*Salix caprea*, clone Boku 04 CZ-024	Heavy metal(loid) contaminated soil	800 g	84 d			X		10 mL 1 x 10^8 CFUs mL^{-1}	De Maria *et al.* (2011)
Rhizospheric and endophytic strains isolated from *S. viminalis* and *S. alba x alba: Rahnella* sp., *Sphingobacterium* sp., *Caulobacter* sp., *Curtobacterium* sp., *Pseudomonas* sp.	*Salix viminalis* and *S. alba x alba*	Former maize field close to a Zn smelter, Cd, Zn and Pb contaminated	4500 g	90 d	X				10^8 CFU mL^{-1}	Janssen *et al.* (2015)
Rhizosphere isolate RX232 and the endophyte EX72	*Salix caprea*, clone Boku 04 CZ-024	Moderately contaminated soil	800 g	84 d			X		10 mL 1 x 10^8 CFUs mL^{-1}	Kuffner *et al.* (2010)
Consortium 1 (German soil): 4 *Streptomyces* sp, 4 *Enterobacter* sp, 1 *Citrobacter* sp, 1 *Psychrobacillu* sp Consortium 2 (Swedish soil): 1 *Acinetobacter* sp, 1 *Rhodococcus* sp, 2 *Arthrobacter* sp, 1 *Pseudomonas* sp, 1 *Lysinibacillus*, 1 *Paenibacillus*	*Agrostis capillaris, Deschampsia flexuosa, Festuca rubra, Helianthus annuus*	(1) One former mining site from Germany, multi-metal(loid)-contaminated (2) One former mining site from Sweden, multi-metal(loid)-contaminated amended with compost		84 d			X		1 x 10^9 CFUs pot^{-1} with 10 mL	Langella *et al.* (2014)

(Continued)

Bacterial inoculant	Plant Host	Soil / substrate type	Amount of soil / substrate	Experiment duration	Inoculation method			Re-inoculation (weeks)	Cellular density	Reference
					Plant	Seed	Substrate			
Pseudomonas sp., *Deltia lacustris*, *Bacillus* sp., *Variovorax boronicumulans*, *Pseudoxanthomonas mexicana*	*Pteris vitatta*	As-contaminated landfill site	3000 g	120 d			X	applied at the beginning and after 2 months	10^8 CFU g^{-1} soil	Lampis *et al.* (2015)
8 *Pseudomonas* sp	*Zea mays* *Helianthus annuus*	Artificially Cu contaminated soil using $CuCl_2$ solution	200 g	30 d	X		X	Applied together	7.5 x 10^8 CFUs mL^{-1}	Li and Ramakrishna (2011)
Ralstonia eutropha, Chryseobacterium humi	*Helianthus annuus*	Agricultural soil artificially contaminated with Zn or Cd	300 g	140 d			X		10 mL 1 x 10^8 CFUs mL^{-1}	Marques *et al.* (2013)
4 *Pseudomonas fluorescens*	*Mirabilis jalapa*	Contaminated soil with Cd, Cr,Cu, Ni, Pb and Zn by a smelter dump		75 d		X			1 - 2 x 10^9 CFUs mL^{-1}	Petriccione *et al.* (2013)
Micrococcus sp.	*Zea mays.* cv. CPDK 888	Garden soil Cd-spiked soil	3000 g	112 d			X	repeated at 2, 4 and 6 weeks	10^6 CFU g^{-1} soil	Sangthong *et al.* (2015)
Root endophyte isolated from *N. caerulescens:Arthrobacter* sp., *Kocuria rhizophila Bacillus* sp. *Microbacterium oxydans*, *Bacillus amyloliquefaciens*	*Noccaea caerulescens, Arabidopsis thaliana*	Ni-rich serpentine soil	750 g	60 d		X			10^8 CFU mL^{-1} (5×10^8 cells mL^{-1})	Visioli *et al.* (2015)
Bacillus subtilis	*Brassica juncea* var. Pusa Bold.	Soil amended with increasing amounts of $NiCl_2$		140 d		X (coated seeds)			2 x 10^5 CFUs $seed^{-1}$	Zaidi *et al.* (2006)

1.5. References

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OBJECTIVES

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Phytoremediation options are receiving increasingly more attention since they are considered to be cost-effective and less invasive than conventional soil remediation techniques, and are able to restore soil fertility and ecosystem functions. In the case of soils with high trace element (TE) content, phytoremediation techniques frequently use high-biomass crops, which are selected according to their tolerance and potential to accumulate/exclude metals in aboveground tissues or to mobilise/immobilise metals in soils. Alternative plant species include those that are able to hyperaccumulate metals in their aboveground tissues.

Trace element (TE)-contaminated lands can be used for the cultivation of high biomass crops for bioenergy purposes thus providing economic benefits for local communities. The use of short rotation coppicing (SRC) of *Salix* spp. and *Populus* spp. and the cultivation of sunflower or tobacco plants can be successfully incorporated into these soil remediation technologies. The use of hyperaccumulator plants for the recovery of valuable metals such as Ni from TE-enriched soils is an alternative for some sites and can also deliver significant economic benefits (such as metal bio-ores).

Studies related to the selection of adequate plant species, the development of conventional agronomic practises and of more novel strategies, such as bioaugmentation, are required if the effectiveness of the remediation process is to be maximised. Furthering our knowledge in these areas will help towards the large -scale implementation of these gentle remediation options. Currently, only a limited number of studies evaluating the feasibility of phytoremediation techniques under field conditions can be found, and there is a clear need for long-term field experiments monitoring the efficiency of these processes if they are to reach full-scale deployment.

Within the field of phytoremediation options for TE-contaminated sites, the objectives of this thesis were firstly, to study the effects of the Ni-hyperaccumulating subspecies of *Alyssum serpyllifolium* on soil Ni availability and fractionation and to characterise the culturable bacterial community associated with this plant, and, secondly, to evaluate the potential application of various bioaugmentation techniques and agronomic practices for improving phytoremediation strategies at both a greenhouse and field scale. To achieve these objectives a series of studies were carried out which can be summarised as follows:

Plant-bacteria-soil system in the rhizosphere of the Ni-hyperaccumulator *Alyssum serpyllifolium* subsp. *lusitanicum*

The objective of the first study (Chapter 3) was to isolate and characterise (both genotypically and phenotypically) culturable rhizobacteria associated with different populations of the two Ni-hyperaccumulating subspecies of *Alyssum serpyllifolium* (subsp. *lusitanicum* and subsp. *malacitanum*) of the Iberian Peninsula and compare these isolates with those in the rhizosphere of non-hyperaccumulating plant species (the Ni-excluder *Dactylis glomerata* and the facultative serpentinophyte *Santolina semidentata*) present in the same ultramafic sites, or in the rhizosphere of the non-hyperaccumulating *Alyssum serpyllifolium* subsp. *serpyllifolium* growing in calcareous soils in Sierra Nevada (S Spain).

The following study (Chapter 4) aimed to evaluate the influence of the Ni-hyperaccumulator *A. serpyllifolium* subsp. *lusitanicum* (which is endemic to the Iberian Peninsula) and the Ni-excluder *Holcus lanatus* (growing at the same serpentine outcrop) on Ni availability in the rhizosphere soil. Nickel availability and fractionation were assessed using conventional extractions, while Ni fluxes in the soil solution and soil replenishment capacity for Ni were assessed using the diffusive gradient in thin films (DGT) technique and DIFS modelling. Chemical imaging of the labile Ni around individual roots of both plant species was achieved using DGT coupled with laser-ablation inductively coupled plasma mass spectrometry (LA-ICP-MS).

Improving the efficiency of phytoremediation techniques using soil amendments and bacterial inoculation

In this study (Chapter 5) the objective was to evaluate the use of composted municipal solid wastes as a means of increasing the phytoextraction efficiency of different Ni hyperaccumulating plants from naturally metal-enriched serpentine soils. The effects of different fertilisation regimes (compost addition or mineral fertilisers) on biomass production and Ni removal of the hyperaccumulators, *Alyssum serpyllifolium* subsp. *lusitanicum* (population of Barazón and Samil), *A. serpyllifolium* subsp. *malacitanum* (population of Sierra Bermeja and Sierra de Aguas), *A. bertolonii* (Imprunetta) and *Noccaea goesingensis* (Redlschlag), were assessed.

The following study (Chapter 6) aimed to explore the interacting effects of organic soil amendments and bacterial inoculants on the phytoextraction efficiency of two high-yielding crops: *Salix caprea* and *Nicotiana tabacum.*

Within this context the objectives were, firstly, to evaluate the ability of the two plant species to accumulate trace elements when cultivated in a mine-soil contaminated with Cd, Pb and Zn, secondly, to assess improvements in the mine-soil properties, plant growth and physiological status, and plant metal accumulation after compost addition, and thirdly, to evaluate the use of plant growth promoting bacteria (PGPB) for improving plant metal extraction when applied alone or in combination with compost.

Following on from this, since inoculation methods were found to differ greatly between studies, and given that these aspects are likely to influence the final effects of such plant-microbial associations, one further study was carried out with the objective of evaluating the effects of different inoculation methods on plant growth and metal accumulation in *Nicotiana tabacum* (Chapter 7). For this, we assessed the effects on plant biomass production and metal accumulation of (i) seed versus soil inoculation, (ii) inocula with different bacterial densities, and (iii) single versus repeated inoculation.

Monitoring short-term effectiveness of phytostabilisation and phytoextraction in field-scale pilot studies

A field trial of aided phytostabilisation was implemented in spring 2011 in Cu-rich mine tailings in the NW of Spain with the objective to establish a healthy plant cover by reducing Cu mobility and improving soil fertility and health. The mine tailings at this site were amended with composted municipal solid wastes and planted with *Salix* spp., *Populus nigra* or *Agrostis capillaris*. Plant growth, nutritive status and metal accumulation, and soil physicochemical and biological properties, were monitored over four years and the main results are presented in Chapter 8.

Finally, in Chapter 9, the main results of a field trial established in 2011 in an abandoned Pb/Zn mine (NW of Spain) evaluating the phytoextraction capacity of the woody trees *Salix viminalis* or *S. atrocinerea*, and the Cd/Zn-hyperaccumulator *Noccaea caerulescens* are presented. The effect of the fertilisation regime (untreated, inorganic fertilisation (NPK) or amendment with composted municipal solid waste) and plant cropping pattern (monoculture vs. intercropping with legumes) on soil physico- and bio-chemical properties, plant growth, nutritive status and metal accumulation were evaluated over two to four years.

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Chapter 3

Rhizobacterial communities associated with the flora of three serpentine outcrops of the Iberian Peninsula.

ABSTRACT

Plant-associated bacteria can improve phytoextraction by increasing plant growth and/or metal uptake. This study aimed to characterise the culturable rhizobacterial community associated with two Ni-hyperaccumulators and to obtain a collection of isolates for application in Ni phytomining. Non-vegetated and rhizosphere soil samples were collected from the Ni-hyperaccumulator *Alyssum serpyllifolium* ssp. *lusitanicum* and *Alyssum serpyllifolium* ssp. *malacitanum*, as well as from non-hyperaccumulating plants (*Dactylis glomerata*, *Santolina semidentata* and *Alyssum serpyllifolium* ssp. *serpyllifolium*). Rhizobacteria were isolated and characterised genotypically and phenotypically. Hyperaccumulating *Alyssum* subspecies hosted higher densities of bacteria compared to either non-hyperaccumulators or non-vegetated soil. In some cases hyperaccumulators showed selective enrichment of Ni-tolerant bacteria. Most bacterial strains belonged to the *Actinobacteria* phylum and presented Ni resistance. Phosphorus-solubilisers were mostly associated with the hyperaccumulators, siderophore-producers with *D. glomerata*, and IAA-producers with both these species. Taxonomic diversity and phenotypic characteristics were soil-, plant species- and plant population-specific. Several strains presented PGP characteristics which could be useful when selecting microorganisms for bioaugmentation trials.

This study formed part of the following publication:

Álvarez-López, V., Prieto-Fernández, A., Becerra-Castro, C., Monterroso, C., Kidd, P.S. (2016). Rhizobacterial communities associated with the flora of three serpentine outcrops of the Iberian Peninsula. *Plant Soil* 403:233-252.

3.1. Introduction

Serpentine soils are derived from ultramafic rocks, where the term ultramafic refers to igneous or metamorphic rocks containing more than 70% of ferromagnesium minerals and a low content in silicon (< 45% SiO_2) (Brooks, 1987). Ultramafic rocks are patchily distributed throughout the world (occupying approximately 1% of the earth's surface area (Proctor, 1999)) and are well-known for their physical and chemical anomalies that present a hostile environment for plant growth. Some common traits include an elevated concentration of Mg and Fe, a low availability of Ca relative to Mg (unfavourable for Ca absorption), a deficiency in essential nutrients such as N, P and K, and high concentrations of potentially phytotoxic trace metals such as Ni, Co and Cr. As a result the plant communities in these areas often present a high number of endemic species (serpentinophytes), and have evolved both morphological and physiological adaptations differentiating them from the flora of adjacent geological substrates (Brooks, 1987). Serpentine flora includes an unusual plant group, the so-called hyperaccumulators, which are able to accumulate extremely high concentrations of Ni in their aerial biomass (>1000 mg kg^{-1} dry weight (DW) matter) (Baker and Brooks, 1989; Chaney *et al.*, 2007). The Iberian Peninsula hosts two subspecies of *Alyssum serpyllifolium* Desf. which are both serpentine-endemic and hyperaccumulators of Ni: *Alyssum serpyllifolium* ssp. *lusitanicum* from Galicia (NW Spain) and Trás-os-Montes (NE Portugal), and *Alyssum serpyllifolium* ssp. *malacitanum* from Andalucía (S Spain).

Nickel-hyperaccumulating plants are considered ideal candidates for application in phytomining, a non-destructive approach for the recovery of high value metals (e.g. Ni) from metal-enriched soils and ores. Plants are cultivated to accumulate trace metals from soils and transport them to the shoots which can then be harvested (Chaney *et al.*, 2007). Bioaugmentation using bacteria associated with hyperaccumulators can improve the plant's capacity to phytoextract metals from soils (Mengoni *et al.*, 2010; Becerra-Castro *et al.*, 2013; Sessitsch *et al.*, 2013). Plant-associated bacteria can enhance plant growth, reduce stress and/or modify soil metal bioavailability (Lebeau *et al.*, 2008; Kidd *et al.*, 2009; Becerra-Castro *et al.*, 2013; Sessitsch *et al.*, 2013; Cabello-Conejo *et al.*, 2014; Glick, 2014). Serpentine soils are a potential source of metal-tolerant (Co, Cr and Ni) bacteria (Pal *et al.*, 2005). Schlegel *et al.* (1991) found bacterial strains isolated from serpentine soils tolerated up to 10-20 mM Ni (in the culture medium), while

strains from other soil types tolerated only 1 mM Ni. Pal *et al.* (2007) found that bacterial strains isolated from the rhizosphere of the Ni-hyperaccumulators *Rinorea bengalensis* and *Dichapetalum gelonoides* tolerated up to 28.9 mM of Ni in the culture medium, while Turgay *et al.* (2012) found that, bacterial strains isolated from Turkish serpentine soils, tolerated up to 34 mM Ni in the growth medium. Furthermore, the rhizosphere bacterial communities associated with Ni-hyperaccumulating plants have been shown to differ from those of non-accumulating plants growing at the same site or of non-vegetated soil, and are also characterised by a higher number of Ni-tolerant bacteria (Schlegel *et al.*, 1991; Mengoni *et al.*, 2001; Abou-Shanab *et al.*, 2003; Idris *et al.*, 2004). This selective enrichment in Ni-tolerant bacteria in the rhizosphere has been correlated with an increase in soil Ni availability (Becerra-Castro *et al.*, 2009). Amongst the culturable Ni-tolerant bacterial strains isolated from serpentine soils and/or associated with Ni-hyperaccumulating plants members of the *Actinobacteria*, *Acidobacteria*, *Chlorobi*, *Firmicutes*, *Verrucomicrobia* and *Proteobacteria* have been described (Mengoni *et al.*, 2001; Oline, 2006; Pal *et al.*, 2007; Turgay *et al.*, 2012).

In this study, the culturable bacterial community associated with different populations of the two Ni-hyperaccumulating subspecies of *Alyssum serpyllifolium* (subsp. *lusitanicum* and subsp. *malacitanum*) of the Iberian Peninsula were characterised. The rhizosphere bacterial communities associated with these plants were compared with those of non-hyperaccumulating plant species (the Ni-excluder *Dactylis glomerata* and the facultative serpentinophyte *Santolina semidentata*) growing at the same sites, as well as with the non-hyperaccumulating *Alyssum serpyllifolium* subsp. *serpyllifolium* growing in calcareous soils (developed over limestone and dolomite) in Sierra Nevada (S Spain). A collection of rhizobacterial isolates was obtained and the strains were characterised both genotypically (BOX-PCR and 16S rDNA partial sequencing) and phenotypically (for their resistance to Ni and plant growth promoting (PGP) traits). We focused on the culturable bacterial community since the global aim is to obtain potentially useful isolates which can be used to improve the Ni phytoextraction capacity of Ni-(hyper)accumulating plant species.

3.2. Materials and methods

Study areas and collection of samples

The study was carried out in five areas of the Iberian Peninsula in which

three subspecies of *Alyssum serpyllifolium* Desf. (Brassicaceae) are found growing. Four of these areas are serpentine outcrops: two sites in Trás-os-Montes (Samil (S; 41°46′48²N; 6°44′47²W) and Morais (M; 41°31′21²N; 06°49′20²W), NW Portugal), one site in Barazón (L) (42°51′09²N; 08°01′15²W, NW Spain) and one in Sierra Bermeja (SB) (36°28′48²N; 05°11′52²W, S Spain). These serpentinitic areas host the two Ni-hyperaccumulating subspecies of *A. serpyllifolium* which are endemic to the Iberian Peninsula: *A. serpyllifolium* subsp. *lusitanicum* Dudley and P. Silva (hereafter referred to as *A. pintodasilvae*) in S, M and L, and *A. serpyllifolium* ssp. *malacitanum* Rivas Goday (hereafter referred to as *A. malacitanum*) in SB. The fifth sampling site was the calcareous dolomitic area of Sierra Nevada (SN) (37°7´21"N; 03°26´53"W, S Spain) where the non-hyperaccumulator *A. serpyllifolium* subsp. *serpyllifolium* Desfontaines grows (hereafter referred to as *A. serpyllifolium*). Both Ni-hyperaccumulating subspecies presented leaf Ni concentrations above 10 g kg^{-1} and the highest Ni accumulation was found in the Spanish population of L (15.5 g kg^{-1}; Table 3.1). For comparative purposes non-hyperaccumulating plants were also collected in some study sites: *Dactylis glomerata* L. was sampled at sites M, S and L, *Santolina semidentata* Hoffmanns. & Link. was sampled at sites M and S.

Trás-os-Montes has a Mediterranean climate, with a mean annual temperature of 12.4ºC and annual precipitation of 720 mm (Carballeira *et al.*, 1983) (Menezes de Sequeira and Pinto da Silva, 1992). Barazón has a European humid-temperate climate with a mean annual temperature of 12.9ºC and mean annual precipitation of 1381 mm (Carballeira *et al.*, 1983). Sierra Bermeja and Sierra Nevada have a Mediterranean oceanic climate (Rivas-Martinez and Rivas-Saenz, 1996-2009), Sierra Bermeja has a mean annual precipitation between 800-1600 mm and mean annual temperature between 14-16ºC (Gómez-Zotano *et al.*, 2014) and Sierra Nevada has a mean temperature of 12ºC and mean annual precipitation between 450 and 1000 mm (Castillo-Martín, 2000) .

At each site, 5 to 7 samples of non-vegetated soil (0-15 cm) or of rhizosphere soil were collected for the isolation of bacterial strains. The whole plant and root system (including root-adhering soil) of each species were collected at late flowering stage, and after gently crushing the root ball the tightly held soil (< 3 mm from the root surface) was considered as rhizosphere soil. Samples were sieved (< 4 mm) and kept at 4ºC until processing. Soil samples are named as follows: L, M, S, SB and SN according to the sampling site (Barazón, Morais, Samil, Sierra Bermeja and Sierra Nevada, respectively); and as A, G and S

according to the plant species (*Alyssum*, *Dactylis* and *Santolina*, respectively). Rhizosphere soil is indicated by an R and the non-vegetated soil by NV.

Elemental analysis of field-collected soils and plant material

Non-vegetated (NV) and rhizosphere samples were air-dried and sieved through a 2-mm stainless steel sieve. Soil pH was measured in H_2O and KCl using a 1:2.5 soil:solution ratio. Total C and N were analysed by combustion with a CHN analyser (Model CHN-1000, LECO Corp., St Joseph, MI). Exchangeable cations (Ca, Mg, Al, Na and K) were extracted with 1 M NH_4Cl and determined by inductively coupled plasma optical emission spectrometry (ICP-OES, model Vista-PRO, Varian). Soils were digested in a 3:1 mixture of concentrated HNO_3:HCl and the total concentrations of Co, Cr and Ni were analysed by ICP-OES. Soil metal availability was evaluated after extraction with water after 24 h shaking using a 1:2.5 soil:H_2O ratio.

Plant material collected in the field was separated in shoots and roots, washed with pressurised tap water followed by deionised water, oven–dried at 45ºC, weighed and ground. Plant shoots (approximately 0.1 g) were digested in a 2:1 concentrated HNO_3:HCl mixture on a hot plate at 120ºC, and the concentration of metals was measured by ICP-OES.

Microbiological analyses

Five grams of fresh rhizosphere soil were suspended in 45 mL sterile sodium hexametaphosphate solution (1%) and shaken for 30 min in an end-over-end shaker. Soil suspensions were diluted in 10-fold series and plated in duplicate onto 284 agar medium (Schlegel *et al.*, 1991) supplemented with 100 μg mL^{-1} of the fungicide cycloheximide. The 284 medium contains (per litre): 6.06 g Tris-HCl, 4.68 g NaCl, 1.49 g KCl, 1.07 g NH_4Cl, 0.43 g Na_2SO_4, 0.2g $MgCl_2.6H_2O$, 0.03 g $CaCl_2.2H_2O$, 0.04 g $Na_2HPO_4.2H_2O$, 10 mL Fe(III)NH_4 citrate solution (containing 48 mg/100mL) plus oligoelements (1.5 mg $FeSO_4.7H_2O$, 0.3 mg H_3BO_4, 0.19 mg $CoCl_2.H_2O$, 0.1 mg $MnCl_2.4H_2O$, 0.08 mg $ZnSO_4.7H_2O$, 0.02 mg $CuSO_4.5H_2O$, 0.036 mg $Na_2MoO_4.2H_2O$) adjusted to pH 7. The medium was supplemented with a mixture of different carbon sources: lactate (0.7g L^{-1}), glucose (0.5g L^{-1}), gluconate (0.7g L^{-1}), fructose (0.5g L^{-1}), and succinate (0.8 g L^{-1}). Densities of metal tolerant bacteria were determined in 284 agar media supplemented with an increasing concentration of Ni (0 mM, 0.5 mM, 1.0 mM, 2.0 mM, 3.0 mM; added as $NiSO_4.6H_2O$). After 7 days incubation at 28ºC, colony

forming units (CFUs) were counted and calculated per gram DW soil. Distinct colony morphotypes from Ni-enriched media associated with each plant species and from each site were sub-cultured at least three times to ensure purity and cryopreserved at −70ºC in culture medium supplemented with 15% (v/v) glycerol.

Phenotypic characterisation of bacterial isolates

Rhizobacterial strains were screened for Ni resistance, the ability to produce biosurfactants, and for various plant growth promoting characteristics: phosphate solubilisation capacity, siderophore production, organic acid production, and indoleacetic acid (IAA) production. Nickel resistance of the strains was tested using 284 agar medium (see above) supplemented with an increasing concentration of Ni (0 mM, 1.0 mM, 2.5 mM, 5.0 mM, 10.0 mM; added as $NiSO_4.6H_2O$) and incubated at 28ºC for 7 days. The Maximal Tolerable Concentration (MTC) of Ni was recorded for each isolate, as the highest Ni concentration tested where the isolate was able to grow. The ability to solubilise inorganic phosphate was assessed in a modified NBRIP agar medium (1.8%) supplied with 5 g L^{-1} of hydroxyapatite and incubated at 28ºC for 5 days (10.0 g glucose, 5.0 g $MgCl_2.6H_2O$, 0.25 g $MgSO_4.7H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$, 0.1 g yeast extract in 1 L deionized water adjusted to pH 7.0; modified from (Nautiyal, 1999)). A clear halo around the bacterial colony indicated solubilisation of mineral phosphate. Yeast extract was added since some strains were unable to grow in yeast-free NBRIP medium. Siderophore production was detected in a modified 284 liquid medium (without Fe) using the Chrome Azurol S (CAS) method described by Schwyn and Neilands (1987). All glassware used in this assay was previously cleaned with 30% HNO_3 followed by washing in distilled water (Cox, 1994). Each isolate was screened for acid production. Single colonies were plated on agar medium containing 0.002% bromocresol purple (per litre medium): 10.0 g glucose, 1.0 g tryptone, 0.5 g yeast extract, 0.5 g NaCl, 0.03 g $CaCl_2.2H_2O$. Colonies forming a yellow halo after 1 day of growth at 28ºC indicated a pH change in the medium and were considered acid producers. The ability of isolated strains to produce IAA was evaluated in liquid medium (5.0 g glucose, 1.0 g $(NH_4)_2SO_4$, 2.0 g K_2HPO_4, 0.5 g $CaCO_3$, 0.5 g $MgSO_4.7H_2O$, 0.1 g NaCl, 0.1 g yeast extract adjusted to pH 7; modified from Sheng *et al.* (2008) supplemented with 0.5 mg mL^{-1} of tryptophan). After 5 days incubation at 28ºC, cultures were centrifuged and the supernatant was incubated with Salkowski reagent for 25 min. The production of IAA was recognised by the presence of red colouring and iso-

lates were considered IAA-producers when the concentration of IAA determined was more than 4 mg L^{-1} culture. Strains were screened for potential biosurfactant production using the qualitative method of Chen *et al.* (2007). The strains were inoculated in 284 liquid medium and cultured overnight at 28ºC, at 150 rpm on a rotary shaker. A 100 µL sample was taken from the supernatant of each strain and added to a microwell of a 96-microwell plate. The plate was then viewed using a backing sheet of paper with a black and white grid. The optical distortion of the grid provided a qualitative assay for the presence of surfactants.

Isolates were classified in different phenotype groups, according to their metal resistance (low Ni tolerance (LT): ≤2.5 mM Ni MTC, or high Ni tolerance (HT): 5-10 mM Ni MTC) and PGP traits (N: non PGP traits producers; A: organic acid-producers; P: P-solubilisers; S: siderophore-producers; T: biosurfactants-producers; H: indoleacetic acid-producers and their possible combinations).

Eighty four out of the 550 rhizobacterial isolates obtained were already phenotypically characterised in a prior study and used to evaluate the potential of the cell-free cultures to mobilise soil Ni under *in vitro* conditions (Becerra-Castro *et al.,* 2011). This previous study targeted only rhizobacterial strains associated with *Alyssum* sp. and their capacity for soil Ni mobilisation.

Genotypic characterisation of bacterial isolates

BOX-PCR genomic DNA profiling

BOX-PCR fingerprinting was used to genotype and group bacterial strains within each isolate collection (L, S, M, SB, SN). Crude cell lysates (colonies suspended in 100 µL and heated to 100ºC for 5 min) were used as DNA templates for BOX-PCR reactions. Box reactions were performed in a total volume of 20 µL containing: 1x Taq buffer (Invitrogen), 1.5 mM $MgCl_2$, 0.1 mM of each dNTP, 0.5U Taq polymerase (Invitrogen), 2 µM of BOX A1R primer (5'- CTACGG-CAAGGCGACGCTGACG-3') (Versalovic *et al.,* 1994), and 2 µL of cell lysate. Thermocycling conditions were: 1 cycle of 94ºC for 5 min; 35 cycles of 1 min at 94ºC; 1.5 min at 50ºC and 8 min at 68ºC; and 1 cycle of 8 min at 68ºC. The obtained PCR products were separated by gel electrophoresis in 1.8% agarose run for 3 h at 3.3 V cm^{-1} gel. Gel images were analysed, using the Pearson correlation coefficient and UPGMA clustering algorithm of the Gel Compar Bionumerics program (Bionumerics Version 6.6, Applied Maths, Belgium). Rhizobacterial isolates were grouped according to their BOX-PCR profiles at a similarity level of

82%. BOX-PCR fingerprints were analysed for each sampling site (L, S, M, SB and SN) and moreover, bacterial strains associated with the Ni-hyperaccumulator *Alyssum* subspecies were analysed separately (LA, SA, MA and SBA). Genetic diversity of isolates was assessed for each plant species and population using the Shannon diversity index ($H' = -\sum(x_i/x_0)\ln(x_i/x_0)$) where x_i is the number of strains per BOX-PCR group for each level of metal tolerance (LT or HT)), and x_0 is the total number of strains in either LT or HT. Isolates within each BOX-PCR group were also classified according to the phenotypes (PGP traits and Ni resistance described above).

DNA extraction and 16S rRNA gene amplification

For DNA extraction, purified strains were grown in 1/10 strength 869 liquid medium (1.0 g tryptone, 0.5g yeast extract, 0.5 g NaCl, 0.1 g glucose, 0.035 g $CaCl_2.2H_2O$ in 1L deionised water (Mergeay *et al.*, 1985)) and genomic DNA was extracted from bacterial cell pellets. Briefly, the method consists of alkaline cell lysis followed by phenol/chloroform/isopropanol alcohol purification. DNA quality was checked by gel electrophoresis on a 0.8% agarose gel. PCR amplification targeting the 16S rRNA gene was carried out using the primers 16S-27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 16S-1492R (5'-TACGGYTACCTTGTTA CGACTT-3') (Lane, 1991). PCR reactions were performed in a total volume of 50 µL containing: 1x Taq buffer (Invitrogen), 2.5 mM $MgCl_2$, 0.1 mM of each dNTP, 1.75U Taq polymerase (Invitrogen), 0.4 µM of each primer, and 1 µL of extracted DNA. Thermocycling conditions were: 2 min at 94ºC; 30 cycles of 1 min at 94ºC; 1 min at 55ºC and 2 min at 72ºC; and 1 cycle of 10 min at 72ºC. PCR products were partially sequenced (between 750 and 1000 bases) using the primer 16S-27F (Lane, 1991). Sequence data were checked using the Chromas v. 1.45 software (Technelysium Pty. Ltd., Australia), uploaded and aligned in the Ribosomal Database Project (RDP; Cole *et al.* (2009)) and assessed for similarity with sequences in the RDP. The sequences used for identification of the culturable strains are available in the EMBL database (www.ebi.ac.uk) under accession numbers HG941722 - HG942010, HE646570 - HE646571, and FN908759 - FN908797 (the latter group were identified and described for Ni mobilisation capacity by Becerra-Castro *et al.* 2011).

Genetic diversity of isolates was assessed for each plant species and population using the Shannon diversity index ($H' = -\sum(x_i/x_0)\ln(x_i/x_0)$) where x_i is the number of strains per genera, and x_0 is the total number of strains.

Statistical analyses

Analyses of variance (ANOVA) were used to detect statistically significant differences between bacterial counts. Comparisons of prevalence of PGP phenotypes (Plant Growth Promoting traits) between plant species were achieved through a chi-squared test or Fisher's exact test when necessary. Statistical analyses were performed using SPSS v.22.0, SPSS Inc., Chicago, IL.

3.3. Results

General soil physicochemical properties

Some general physicochemical properties of the soils at each site are given in Table 3.1, as well as the total concentration of Ni in aboveground shoots of each plant species studied. All four serpentine soils were characterised by pH values close to neutrality, a low C and N content, and a predominance of Mg in the exchange complex, and Ca/Mg quotients of < 1. In contrast, SN soil presented alkaline pH values and Ca dominating the cation exchange complex, higher organic matter content and C/N ratio. Total Ni concentrations were higher in serpentine soils than the calcareous SN soil. Values were similar in the Portuguese sites (S and M; 3.03 and 2.72 g kg^{-1}, respectively) and lower in the L and SB soils (2.20 and 2.10 g kg^{-1}, respectively). In contrast, total Ni in SN calcareous soil was only 0.03 mg kg^{-1}. In accordance, labile Ni (water-soluble) was highest in the S site. Water-soluble Ni concentrations were higher in the rhizosphere soil of the M population of the Ni-hyperaccumulator compared to the non-hyperaccumulators *D. glomerata* (1.2-fold lower) and *S. semidentata* (2.2-fold lower) or the non-vegetated soil (3.1-fold lower). Also the rhizosphere soil of the Ni-hyperaccumulator in SB presented a higher concentration of water-soluble Ni than the non-vegetated soil (2.1-fold lower).

Culturable bacterial densities and the rhizosphere effect

The densities of culturable bacteria in non-vegetated and rhizosphere soils of the different plant species and sites are presented in Table 3.2. Bacterial densities in non-vegetated serpentine soils did not differ significantly, and ranged from 3.7 x 10^7 CFU g^{-1} DW soil in L to 7.9 x 10^7 CFU g^{-1} DW soil in SB (Tukey post-hoc test showed no differences between NV serpentine soils; $p > 0.05$). On the other hand, bacterial densities in non-vegetated soils of SN were at least 10-fold higher than those of the serpentine sites (5.2 x 10^8 CFU g^{-1} DW soil).

Table 3.1. Leaf Ni accumulation and soil physicochemical characteristics (mean ±SE) at the different study sites. L (Barazón, NW Spain), S and M (Samil and Morais, NE Portugal), SB and SN (Sierra Bermeja and Sierra Nevada, S Spain).

	L	S	M	SB	SN
Leaf Ni concentration (g kg^{-1})					
Alyssum	15.46 ± 0.72	11.02 ± 0.12	10.88 ± 0.74	10.39 ± 0.77	< LOQ
Dactylis	0.06 ± 0.01	0.35 ± 0.07	0.14 ± 0.03	-	-
Santolina	-	0.19 ± 0.04	0.23 ± 0.02	-	< LOQ
Soil physicochemical properties					
pH (H_2O)	7.2 ± 0.1	6.9 ± 0.1	6.9 ± 0.1	7.4 ± 0.0	8.0 ± 0.0
pH (KCl)	6.0 ± 0.1	6.3 ± 0.1	6.2 ± 0.1	5.6 ± 0.0	7.2 ± 0.0
Total C (%)	1.28 ± 0.11	3.05 ± 0.24	3.0 ± 0.27	2.42 ± 0.10	9.73 ± 0.05
Total N (%)	0.10 ± 0.01	0.28 ± 0.02	0.26 ± 0.02	0.21 ± 0.00	0.46 ± 0.01
C/N	12.7 ± 0.6	10.8 ± 0.4	11.5 ± 0.3	11.5 ± 0.4	21.2 ± 0.2
CEC ($cmol_c$ kg^{-1})	11.8 ± 0.3	23.2 ± 1.1	22.4 ± 0.5	25.9 ± 0.5	23.9 ± 0.5
Ca/Mg	0.3 ± 0.0	0.6 ± 0.1	0.3 ± 0.0	0.5 ± 0.0	2.9 ± 0.0
Total Ni (g kg^{-1})	2.20 ± 0.20	3.03 ± 0.12	2.72 ± 0.04	2.10 ± 0.03	0.03 ± 0.0
Water soluble Ni (mg kg^{-1})					
Non-vegetated soil	0.97 ± 0.12	1.40 ± 0.23	0.81 ± 0.03	0.33 ± 0.02	< LOQ.
Rhizosphere soil					
Alyssum	0.74 ± 0.17	1.09 ± 0.30	2.52 ± 0.58	0.69 ± 0.12	< LOQ
Dactylis	1.42 ± 0.31	1.18 ± 0.21	2.12 ± 1.63	-	< LOQ
Santolina	-	1.03 ± 0.09	1.13 ± 0.18	-	-

LOQ: limit of quantification; 0.025 mg kg^{-1} for water soluble Ni and 0.005 g kg^{-1} for leaf Ni concentration.

The effect of the plant on bacterial densities (rhizosphere effect) was assessed using the R / NV ratio (CFUs g^{-1} rhizosphere soil / CFUs g^{-1} non-vegetated soil) (Table 3.2). Bacterial densities were higher (albeit not always significantly) in the rhizosphere of all the plant species compared to non-vegetated soil, with the exception of *D. glomerata* in M (R / NV of 0.9). The highest densities were always associated with the *Alyssum* subspecies, and this was the case for all five populations. The increase in bacterial densities in the rhizosphere ranged from 1.3-fold in the S population of *D. glomerata* to 4.0-fold in the L population of *A. pintodasilvae*. The most pronounced rhizosphere effect was observed in the

L population of *A. pintodasilvae* (R / NV ratio of 4; $p < 0.001$), where densities were up to one order of magnitude higher in the rhizosphere soil compared to non-vegetated soil. On the other hand, the biggest differences in rhizosphere bacterial densities between different plant species growing at the same site were observed in M. In this case the bacterial densities associated with *A. pintodasilvae* were 1.7 x 10^8 CFU g^{-1} soil, which was 3.4- and 2.3-fold higher than those found in the rhizosphere soil of *D. glomerata* (7.5 x 10^7 CFU g^{-1} soil; $p < 0.05$) and *S. semidentata* (5.0 x 10^7 CFU g^{-1} soil; $p < 0.001$), respectively. In fact, the rhizosphere bacterial densities associated with *S. semidentata* from this site (5.0 x 10^7 CFU g^{-1} soil) were similar to those determined in non-vegetated soil (5.5 x 10^7 CFU g^{-1} soil).

Nickel resistance of the culturable bacterial community

Bacterial densities of soils (NV and rhizospheric) plated in culture media supplemented with Ni decreased with an increase in the medium Ni concentration, and this was observed for all plant species and populations (Table 3.2). Compared to the total bacterial population, densities of Ni-resistant bacteria in serpentine soils were reduced by at most one order of magnitude at the highest metal concentration tested. In contrast, in SN, densities were reduced by up to three orders of magnitude: in non-vegetated soil, densities decreased from 5.2 x 10^8 CFU g^{-1} soil (0 mM Ni) to 6.0 x 10^5 CFU g^{-1} soil at the highest Ni concentration (3 mM) and in *A. serpyllifolium* rhizosphere soil they fell from 1.1 x 10^9 CFU g^{-1} soil (0 mM Ni) to 1.6 x 10^6 CFU g^{-1} soil (3 mM Ni).

As observed for the total culturable bacterial population, the Ni-resistant population (CFU g^{-1} soil) was highest in the rhizosphere of the Ni-hyperaccumulator compared to non-vegetated soil, these plants also tended to present higher R / NV ratios compared to non-accumulators. A strong rhizosphere effect (R / NV ratios < 2) was not observed for any population of *S. semidentata* or *D. glomerata* (except for the L population of *D. glomerata*; Table 3.2). The M population of *A. pintodasilvae* hosted up to 6-fold higher densities of Ni-resistant bacteria in culture medium with 2 mM Ni compared to non-vegetated soil (R / NV of 6.1), while the R / NV ratios for the same population of *D. glomerata* and *S. semidentata* were at most 1.5. At the same site the abundance of Ni tolerant bacteria was also significantly higher in the rhizosphere of the hyperaccumulator than in the rhizosphere of *S. semidentata* or *D. glomerata*. In the SB population of

Table 3.2. Colony forming units per gram dry weight soil (CFUs * 10^6 g^{-1} ± SE) in non-vegetated and rhizosphere soils, and the ratio rhizosphere (R): non-vegetated soil (NV), of the serpentine (Barazón, Samil, Morais, S. Bermeja) and the calcareous soils (S. Nevada). Different letters indicate significant differences between plant species and non-vegetated soil at the same site ($p < 0.05$). NV (non-vegetated soil), LA (*A. pintodasilvae* from Barazón), LG (*D. glomerata* from Barazón), SA (*A. pintodasilvae* from Samil), SG (*D. glomerata* from Samil), SS (*S. semidentata* from Samil), MA (*A. pintodasilvae* from Morais), MG (*D. glomerata* from Morais), MS (*S. semidentata* from Morais), SBA (*A. malacitanum* from S. Bermeja) and SNA (*A. serpyllifolium* from S. Nevada).

	Control		Ni 0.5 mM		Ni 1 mM		Ni 2 mM		Ni 3 mM	
	CFUs * 10^6	R/NV	CFUs * 10^6	R/NV	CFUs * 10^6	R/NV	CFUs * 10^6	R/NV	CFUs * 10^6	R/NV
Barazón										
NV	37.4 ± 28.3 *a*		20.6 ± 16.1 *a*		10.6 ± 10.3 *a*		5.2 ± 6.5 *a*		2.7 ± 3.3 *a*	
LA	149.5 ± 20.1 *b*	4.0	68.1 ± 14.3 *b*	3.3	40.3 ± 10.4 *b*	3.8	15.6 ± 6.3 *a*	3	7.3 ± 3.1 *a*	2.7
LG	115.2 ± 20.3 *b*	3.1	44.4 ± 7.8 *ab*	2.2	30.8 ± 6.5 *ab*	2.9	17.1 ± 3.4 *a*	3.3	9.1 ± 2.1 *a*	3.3
Samil										
NV	50.9 ± 6.4 *a*		47.0 ± 6.3 *a*		28.3 ± 2.3 *a*		15.1 ± 1.1 *a*		7.6 ± 1.3 *a*	
SA	98.8 ± 10.0 *b*	1.9	49.1 ± 9.2 *a*	1	36.8 ± 6.3 *a*	1.2	23.4 ± 6.1 *a*	1.6	15.1 ± 3.2 *b*	2.2
SG	75.7 ± 13.4 *ab*	1.3	42.8 ± 5.1 *a*	0.9	25.6 ± 1.8 *a*	0.9	16.2 ± 3.8 *a*	1.1	10.9 ± 0.6 *ab*	1.4
SS	67.5 ± 5.3 *ab*	1.5	47.8 ± 6.0 *a*	1	35.0 ± 5.8 *a*	1.2	21.6 ± 2.9 *a*	1.4	11.8 ± 1.7 *ab*	1.6
Morais										
NV	54.6 ± 9.6 *a*		30.2 ± 6.0 *a*		22.9 ± 2.8 *a*		10.2 ± 1.1 *a*		5.4 ± 1.4 *a*	
MA	171.6 ± 19.7 *b*	3.1	120.1 ± 14.5 *b*	4	97.6 ± 15.9 *b*	4.3	62.0 ± 6.7 *b*	6.1	20.3 ± 4.3 *b*	3.7
MG	74.8 ± 7.0 *a*	0.9	24.8 ± 3.3 *a*	0.8	18.2 ± 1.4 *a*	0.8	10.0 ± 2.4 *a*	1	5.5 ± 1.4 *a*	1
MS	50.0 ± 4.8 *a*	1.4	23.7 ± 0.4 *a*	0.8	29.6 ± 2.5 *a*	1.3	14.9 ± 5.3 *a*	1.5	9.3 ± 4.7 *a*	1.7
S. Bermeja										
NV	79.4 ± 7.8 *a*		25.8 ± 1.0 *a*		15.3 ± 1.8 *a*		5.0 ± 0.4 *a*		2.3 ± 0.1 *a*	
SBA	237.8 ± 40.6 *b*	3.0	96.1 ± 9.0 *b*	3.7	63.6 ± 14.2 *b*	3.7	24.6 ± 4.7 *b*	5	11.2 ± 1.6 *b*	4.9
S. Nevada										
NV	520.0 ± 33.4 a		16.1 ± 3.0 *a*		4.6 ± 1.4 *a*		1.8 ± 0.6 *a*		0.6 ± 0.2 *a*	
SNA	1083.0 ± 179.9 *b*	2.1	41.0 ± 9.5 *a*	2.5	12.7 ± 2.4 *b*	2.8	2.0 ± 0.7 *a*	1.2	1.6 ± 0.6 *a*	2.6

A. malacitanum Ni-resistant bacterial densities were 5-fold higher than those of non-vegetated soil in media supplemented with either 2 or 3 mM Ni (Table 3.2).

In most cases the presence of the plant did not affect bacterial Ni tolerance. For example, in the case of L no differences were observed between the % Ni-resistant bacteria associated with the different plant species and the non-vegetated soil. In S, at the two lower concentrations of Ni (0.5 and 1 mM) the rhizosphere of all three plant species harboured a lower % of Ni-tolerant bacteria compared to non-vegetated soil. However, in the case of the Ni-hyperaccumulators *A. pintodasilvae* (M population) and *A. malacitanum* (SB population), these plants harboured a higher proportion of Ni-tolerant bacteria than their corresponding non -vegetated soils: increasing from 19.6% in non-vegetated soil to 36.8% in the rhizosphere of the M population of *A. pintodasilvae* (at 2 mM Ni) and from 19.5 to 27% in the SB population of *A. malacitanum* (at 1 mM Ni) (Table 3.2).

Phenotypic characteristics of the bacterial isolates

A total of 550 different colony morphotypes (74 associated to LA, 64 to SA, 54 to MA, 95 to SBA, 48 to LG, 41 to SG, 44 to MG, 43 to SS, 45 to MS, and 42 to SNA) were isolated and analysed for their plant growth promoting traits and Ni tolerance (MTC). Figure 3.1a summarises the frequency of PGP traits found amongst the rhizobacterial isolates. All of the plant species harboured rhizobacteria which were capable of producing organic acids. This was in fact the most common trait amongst the collection of rhizobacteria: 29.3% of the isolated strains were characterised as organic acid-producers. Depending on the plant species or population between 12 and 61% of the isolates were organic acid-producers. The highest numbers of organic acid-producers were associated with *S. semidentata* (51.2 to 62.2% of the isolates from this plant species showed this trait), followed by the *Alyssum* subspecies (24.3 to 37.5%) and finally, *D. glomerata* (12.2 to 15.9%). The ability to produce IAA was also present in all plant species and represented 23.1% of the total of number of isolates. IAA-producers were most common in the L, S and M serpentine sites. Similar proportions of IAA-producers were found for isolates of *A. pintodasilvae* and *D. glomerata* in L (31.1-39.6%) and S (21.9-24.4%) populations. In M a higher% of IAA-producers were found amongst isolates of *A. pintodasilvae* (44.4%), and a similar% was obtained from isolates of either *D. glomerata* (20.5%) or *S. semidentata* (17.8%). IAA-producers were less frequently associated with either *A. malacitanum* from SB (9.5% of isolates) or *A. serpyllifolium* from SN (14%). The mean production of IAA (mg

L^{-1}) by strains varied from 5.3 in SA to 48.0 in LG, however, a high variability was found between isolates from each site. The maximum IAA production was recorded in the population of *D. glomerata*, from Barazón, where three isolates showed IAA production higher than 200 mg L^{-1} (247.9 mg L^{-1} for strain LG105; 315.9 mg L^{-1} for strain LG120 and 211.4 mg L^{-1} for strain LG121). In addition, one strain associated with the grass species in Samil also showed IAA production (up to 118.2 mg L^{-1}; strain SG5), while in the case of the hyperaccumulator, the maximum IAA production was recorded in *A. pintodasilvae* from Barazón (136.2 mg L^{-1}; strain LA 24).

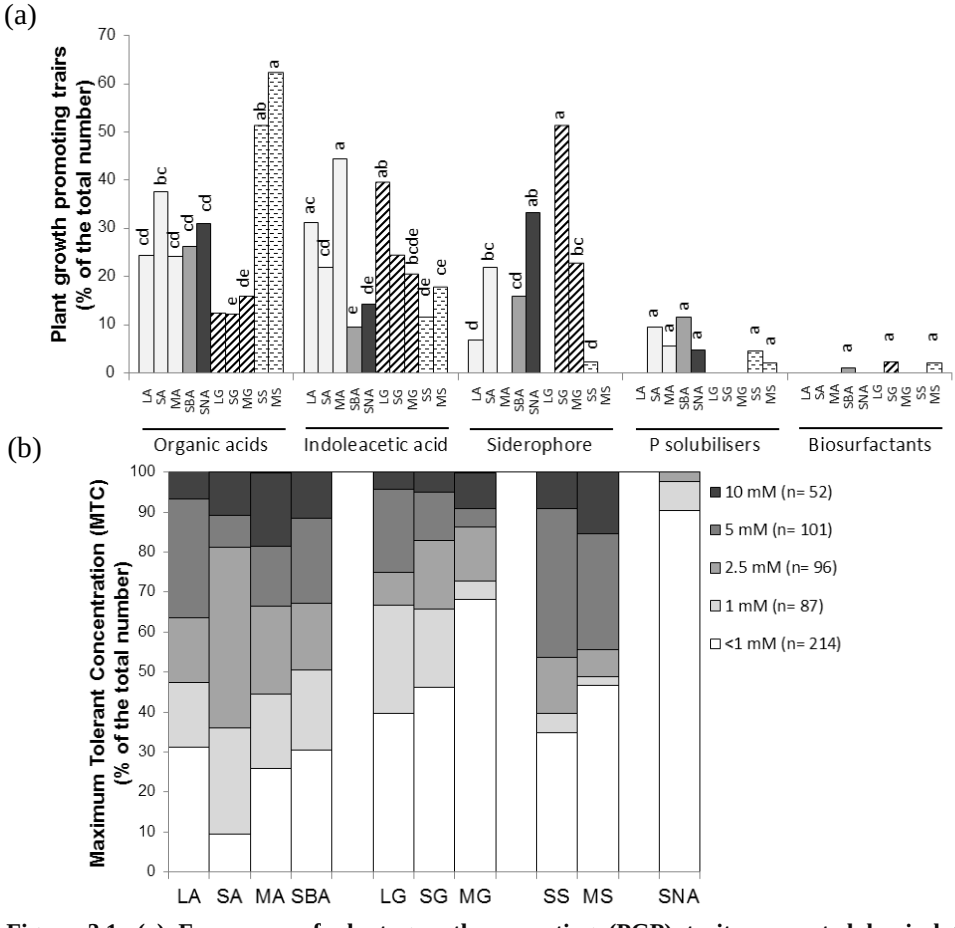


Figure 3.1. (a) Frequency of plant growth promoting (PGP) traits presented by isolates obtained from the different populations of each plant species (presented as a% of the total number of isolates per group). (b) Maximal Tolerable Concentration (MTC) of bacteria isolated from the different rhizosphere soils (presented as a percentage of the total number of isolates in each group). The metal resistance was tested using 284 agar medium supplemented with 1, 2.5, 5 or 10 mM Ni added as $NiSO_4$ and incubated at 28ºC for 7 days. LA (*A. pintodasilvae* from Barazon), LG (*D. glomerata* from Barazón), SA (*A. pintodasilvae* from Samil), SG (*D. glomerata* from Samil), SS (*S. semidentata* from Samil), MA (*A. pintodasilvae* from Morais), MG (*D. glomerata* from Morais), MS (*S. semidentata* from Morais), SBA (*A. malacitanum* from S. Bermeja) and SNA (*A. serpyllifolium* from S. Nevada).

Siderophore-producing strains were found in the rhizosphere of all the *Alyssum* populations studied except in *A. pintodasilvae* from M. The frequency of isolates showing this characteristic varied between 6.8% in L and 33.3% in SN. The siderophore-producing ability was abundant in isolates from the rhizosphere of *D. glomerata* collected in M (51.2%) and S (21.7%). In contrast, in the rhizosphere of *S. semidentata* these were much less frequent (only 2.3% of isolates in S). The abilities to solubilise mineral phosphate or to produce biosurfactants were relatively rare traits amongst the bacterial isolates, representing 4.5 and 0.5% of the total number of the isolates, respectively. P solubilisers were not found in isolates from *D. glomerata* but were isolated from the rhizosphere of the *Alyssum* and *S. semidentata* populations (except in L). In *Alyssum* between 4.8 and 11.6% of the isolates were able to solubilise P while in *S. semidentata* this characteristic represented 2.2 and 4.6% of the isolates. Biosurfactant production was only detected amongst 3 rhizobacteria isolated from *A. malacitanum* (SB), *S. semidentata* (of M) and *D. glomerata* (of S), and the percentage was never greater than 3%.

Figure 3.1b shows the frequency of isolates (as a % of the total number of isolates in each group) for each MTC. All of the serpentine plant species harboured rhizobacteria capable of growing in all the tested Ni concentrations. However, the level of Ni tolerance depended on both the soil type and the plant species. About 90% of rhizobacterial isolates associated with the non-hyperaccumulator (SN) were only able to grow in medium without Ni (MTC < 1 mM), while for the plant species growing in the serpentine sites from 4.2 to 18.5% of rhizobacteria presented a MTC of 10 mM. Of the rhizobacterial isolates associated with non-hyperaccumulators species growing on serpentine sites the majority presented MTC values < than 1 mM Ni. For instance, most of the isolates associated with the Ni-excluder *D. glomerata* (66-73%) did not tolerate Ni concentrations $\geq$2.5 mM, while in the rhizosphere of the Ni-hyperaccumulators this percentage ranged from 36 to 51%.

Within the Ni-hyperaccumulators, only 9.4% of strains associated with the S population of *A. pintodasilvae* were unable to grow in the presence of Ni in the growth medium. The same population presented the highest percentage (45.3%) of associated microorganisms with a MTC of 2.5 mM Ni. While the L population presented the highest number of isolates with an MTC of 5 mM (30%) and the M population of 10 mM (18.5%). Similarly, it was the Ni-hyperaccumulators which presented the highest numbers of associated bacteria with an MTC of 10 mM Ni (6.8, 10.9 and 18.5% in the L, S and M sites, respectively). Nonetheless, a high

percentage (at times similar or even higher than the Ni-hyperaccumulators) of isolates associated with the non-hyperaccumulator *S. semidentata* also presented elevated Ni MTC values (up to 5 or 10 mM).

Table 3.3 summarises the frequency of each phenotype within the collection of isolates. The most frequent phenotype was LT-N (47% in L, 25% in M, 28% in S, 31% in SB and 38% in SN), which represents low Ni tolerance (up to 2.5 mM Ni MTC) and no PGP trait. This was followed in the serpentine sites, by HT-N (high Ni tolerance but no PGP trait) (18% in L, 16% in M, 12% in S, 23% in SB) and by LT-A in the SN site (17%). Phenotypes A and H (which correspond with organic acid- and IAA-producers respectively), were also frequently found in LT strains (up to 18% in L) but were less often represented by HT strains (up to 10% in S). Phenotypes presenting three PGP traits (phenotypes APH, ASH and PSH) were rarely found (at most 2% of strains). In general, all phenotypes were represented in both ranges of metal tolerance (LT and HT), except phenotypes T and APH which were exclusive to HT isolates and phenotypes AP, PH, ST and PSH which were only present in LT isolates. Strains isolated from L showed a maximum of two PGP traits (HT-SH, siderophore- and IAA-producers; Table 3.3). Whereas in S, M and SB several strains with more than two PGP traits were found (phenotypes APH, ASH and PSH); and these were mainly associated with the Ni-hyperaccumulator. Rhizobacterial strains associated with *Alyssum* ssp. generally presented a higher diversity in phenotypes than the other plant species. For example, in the L population *A. pintodasilvae* strains were allocated into 12 different phenotypes, while only five phenotypes were found in rhizobacteria from the gramineae growing in the same site, and none of these were specific to the grass species. In the Portuguese (M and S) populations, phenotype distribution depended on the plant species. In M, phenotypes LT-AP and HT-APH were associated with *A. pintodasilvae*, while HT-S, LT-AS and LT-SH (all of which include siderophore-producers) with *D. glomerata*, and finally, HT-T with *S. semidentata*. In S, the 18 phenotypes were distributed among the plant species as follows: *A. pintodasilvae* (15 groups) > *D. glomerata* (10) > *S. semidentata* (9); four of the groups were specific to the hyperaccumulator (LT-PH, LT-PSH, HT-AS, and HT-ASH), two to *D. glomerata* (LT-ST and LT-ASH) and only one (HT-APH) was specific to *S. semidentata*. In SB and SN, strains associated with *Alyssum* sp. were represented by ASH and PH phenotypes, respectively.

Table 3.3. Frequency of bacterial phenotypes associated with the different plant species (A, *Alyssum*; G, *Dactylis*; Sa, *Santolina*) of each site: L (Barazón), S (Samil), M (Morais), SB (S. Bermeja) and SN (S. Nevada).

	L			S				M				SB		SN	
Phenotypic group	%	A (%)	G (%)	%	A (%)	G (%)	Sa (%)	%	A (%)	G (%)	Sa (%)	%	A (%)	%	A (%)
LT N	**47**	21	26	28	14	7	8	25	7	13	4	**31**	31	**38**	38
HT N	**18**	11	7	12	3	4	4	16	8	2	6	**23**	23		
LT A	**7**	3	3	11	5	1	4	18	4	2	11	**12**	12	**17**	17
HT A	**5**	3	2	10	2		8	4		1	3	**2**	2		
LT P				3	2		1	1	1		1	**4**	4	**5**	5
HT P												**4**	4		
LT S	**1**	1		8	1	7	1	4	1	3		**5**	5	**17**	17
HT S	**2**	2		1	1	1		1		1				**2**	2
LT T															
HT T								1			1	**1**	1		
LT H	**9**	9		5	3	1	1	14	9	5		**3**	3	**2**	2
HT H	**4**	3	2					4	4		1				
LT AP								1	1			**1**	1		
HT AP															
LT AS	**1**	1		6	5	1		1		1		**6**	6	**7**	7
HT AS				1	1										
LT AH	**5**	5		5	3		2	4	2		1	**3**	3	**5**	5
HT AH	**2**	2						4	1		4				
LT PH				1	1										
HT PH															
LT ST				1		1									
HT ST															
LT SH				5	1	4		3		3				**5**	5
HT SH	**1**	1													
LT APH															
HT APH				1			1	1	1						
LT ASH				1		1						**2**	2	**2**	2
HT ASH				1	1										
LT PSH				1	1							**1**	1		
HT PSH															
Number of strains		74	48		64	41	43		54	44	45		95		42

LT (low Ni tolerance ≤ 2.5 mM Ni MTC), HT (high Ni tolerance 5-10 mM Ni MTC).N: no PGP traits; A: organic acid-producers; P: P-solubilisers; S: Siderophore-producers; T: biosurfactants-producers; H: indoleacetic acid-producers and combinations.

Identification and genotypic diversity of culturable rhizobacteria

BOX-PCR profiles

A BOX-PCR profile was obtained for 538 out of the 550 isolated bacterial strains. Profiles were analysed on a site-by-site basis (for each plant species present) and for all the Ni-hyperaccumulating *Alyssum* species together (Table 3.4 and Table 3.5). Table 3.6 presents a summary of the total number of BOX-PCR groups obtained in each of these analyses and the distribution of bacterial isolates amongst these groups for each site (Table 3.6a) and plant species (Table 3.6b). In the case of L the BOX-PCR profiles of isolates were distributed amongst 19 groups, strains associated with the Ni-hyperaccumulator *A. pintodasilvae* were distributed amongst 13 groups and those of *D. glomerata* in 13 groups. Twelve of these BOX-PCR groups are represented exclusively by strains of either *A. pintodasilvae* or *D. glomerata* (groups L01-L02, L04, L07-L08, L11-L12, L14 and L16-L19), while seven groups are made-up of isolates from both species (Table 3.4). In the case of S, the BOX-PCR profiles of isolates from *A. pintodasilvae* and *S. semidentata* were distributed amongst 19 and 9 groups each, while for *D. glomerata* isolates were distributed amongst only 4 groups. Twelve groups were exclusively made up of isolates associated with *A. pintodasilvae* (S01, S02, S07, S09, S11, S12, S15, S16, S17, S18, S20 and S21), three groups with isolates of *S. semidentata* (S10, S19 and S22), and some groups (S04, S08 and S13) were composed of isolates from both the Ni-hyperaccumulator and *S. semidentata*. There were no groups solely represented by isolates of *D. glomerata* (Table 3.5). In the case of plant populations of M, isolates were distributed in 24 BOX-PCR groups; *A. pintodasilvae* isolates were distributed amongst 17 groups, S. *semidentata* amongst 17 groups and *D. glomerata* amongst 8 groups. The groups are generally represented by isolates from all three plant species, with some exceptions being group M01 (solely represented by isolates of *D. glomerata*), M02, M10, M19, M23-M24 (each represented by a single isolate of *A. pintodasilvae*), M03, M14, M16-18 and M22 (isolates of *S. semidentata*) (Table 3.4). Rhizobacterial strains isolated from *A. malacitanum* (from SB) and from *A. serpyllifolium* (from SN) were allocated into 18 and 10 BOX-PCR groups, respectively (Table 3.4).

Table 3.4. BOX-PCR groups and phenotypic characteristics of rhizobacterial strains of *Alyssum* sp. (A), *Dactylis glomerata* (G) isolated from Barazón (L), Samil (S), Morais (M), S. Bermeja (SB) and S. Nevada (SN). OA, organic acid-producer; P, phosphate solubilisation capacity; Sf, biosurfactant-producer; Sd, siderophore-producer; IAA, indoleacetic acid-producer. In Barazón, BOX PCR groups were based on profiles obtained for 119 bacterial strains (72 correspond with LAR, 47 with LGR), In Samil, BOX PCR groups were based on profiles obtained for 144 bacterial strains (61 associated with SAR, 41 with SGR, 42 with SSR). In Morais, BOX PCR groups were based on profiles obtained for 139 bacterial strains (53 associated with MAR, 41 with MGR and 45 with MSR). In S. Bermeja, BOX PCR groups were based on profiles obtained for 94 bacterial strains and in S. Nevada groups were based on profiles obtained for 42 bacterial strains. The number of strains for each phenotype and plant are given for each BOX group.

Box Group	OA	P	Sd	Sf	IAA	Phenotype	A (nº)	G (nº)
BARAZÓN								
L01	-	-	-	-	-	LT N		1
	+	-	-	-	-	LT A		1
L02	-	-	-	-	-	HT N		2
	-	-	-	-	+	HT H		1
L03	-	-	-	-	-	LT N	5	10
	+	-	-	-	-	LT A	1	
	-	-	+	-	-	LT S	1	
	-	-	-	-	+	LT H	2	
	+	-	-	-	+	LT AH	1	
	-	-	-	-	-	HT N	1	3
	+	-	-	-	-	HT A	1	
	-	-	+	-	-	HT S	1	
	-	-	-	-	+	HT H	1	
	+	-	-	-	+	HT AH	1	
L04	-	-	-	-	+	LT H	2	
	+	-	-	-	+	LT AH	1	
L05	-	-	-	-	-	HT N		1
	-	-	+	-	+	HT SH	1	
L06	-	-	-	-	+	LT H	1	
	-	-	-	-	-	HT N	1	1
L07	+	-	-	-	-	HT A	1	
L08	-	-	-	-	-	LT N		1
L09	-	-	-	-	-	LT N	11	7
	+	-	-	-	-	LT A		1
	-	-	-	-	+	LT H	3	
	+	-	-	-	+	LT AH	2	
	-	-	-	-	-	HT N	4	1
	+	-	-	-	-	HT A	1	2
	+	-	-	-	+	HT AH	1	
L10	-	-	-	-	-	LT N	5	3
	+	-	-	-	-	LT A	3	1
	-	-	-	-	+	LT H	1	
	+	-	+	-	-	LT AS	1	
	+	-	-	-	+	LT AH	1	
	-	-	-	-	-	HT N	2	
L11	-	-	-	-	-	LT N		1
	+	-	-	-	-	LT A		1
L12	-	-	-	-	-	LT N	1	

Box Group	OA	P	Sd	Sf	IAA	Phenotype	A (nº)	G (nº)
L13	-	-	-	-	-	LT N	3	5
	-	-	-	-	+	LT H	1	
	-	-	-	-	-	HT N	4	
	-	-	+	-	-	HT S	1	
	-	-	-	-	+	HT H		1
L14	+	-	-	-	+	LT AH	1	
	-	-	-	-	+	HT H	1	
L15	-	-	-	-	-	LT N		1
	-	-	-	-	-	HT N	1	
L16	-	-	-	-	-	LT N		1
L17	-	-	-	-	-	LT N		1
L18	+	-	-	-	-	HT A	1	
L19	-	-	-	-	+	LT H	1	

Box Group	**OA**	**P**	**Sd**	**Sf**	**IAA**	**Phenotype**	**A (nº)**	**G (nº)**	**Sa (nº)**
SAMIL									
S01	+	-	-	-	-	HT A	1		
S02	-	-	-	-	+	LT H	1		
S03	+	-	-	-	-	LT A			1
	-	+	+	-	+	LT PSH	1		
S04	-	-	-	-	-	LT N			1
	+	-	+	-	-	LT AS	2		
S05	-	-	-	-	-	LT N	4	3	
	+	-	-	-	-	LT A	3		1
	-	+	-	-	-	LT P	1		
	-	-	+	-	-	LT S	1		
	-	-	-	-	+	LT H	2		
	+	-	+	-	-	LT AS	4		
	+	-	-	-	+	LT AH	2		
	-	-	+	-	+	LT SH	2	2	
	-	-	-	-	-	HT N		1	2
	+	-	-	-	-	HT A	1		4
	+	-	+	-	+	HT ASH	1		
S06	-	-	-	-	-	LT N	5	7	7
	+	-	-	-	-	LT A	2	2	1
	-	+	-	-	-	LT P	1		
	-	-	+	-	-	LT S		10	1
	-	-	-	-	+	LT H		2	
	+	-	+	-	-	LT AS		2	
	+	-	-	-	+	LT AH	1		2
	-	-	+	+	-	LT ST		1	
	-	-	+	-	+	LT SH		3	
	+	-	+	-	+	LT ASH		1	
	-	-	-	-	-	HT N	3	5	2
	+	-	-	-	-	HT A	1		6
	-	-	+	-	-	HT S	1	1	
	+	+	-	-	+	HT APH			1
S07	-	-	-	-	+	LT H	1		
S08	-	-	-	-	-	LT N	1		1
S09	-	-	-	-	-	LT N	1		
S10	-	+	-	-	-	LT P			1
S11	+	-	-	-	-	LT A	1		
	+	-	+	-	-	LT AS	1		

(continued)

Box Group	OA	P	Sd	Sf	IAA	Phenotype	A (nº)	G (nº)	Sa (nº)
S12	-	-	-	-	-	LT N	1		
	+	-	-	-	-	LT A	1		
S13	-	-	-	-	-	LT N			1
	+	-	-	-	+	LT AH			1
	-	+	-	-	+	LT PH	1		
	-	-	-	-	-	HT N	1		
	+	-	-	-	-	HT A			1
S14	-	-	-	-	-	LT N	2		1
	+	-	-	-	-	LT A			2
	+	-	-	-	+	LT AH	1		
	-	-	+	-	+	LT SH		1	
	-	-	-	-	-	HT N			1
	+	-	+	-	-	HT AS	1		
S15	-	-	-	-	-	LT N	1		
	-	+	-	-	+	LT PH	1		
S16	-	-	-	-	-	LT N	1		
S17	-	-	-	-	-	LT N	1		
	-	+	-	-	-	LT P	1		
S18	+	-	-	-	-	LT A	1		
S19	+	-	-	-	-	LT A			1
	-	-	-	-	+	LT H			1
	-	-	-	-	-	HT N			1
S20	-	-	-	-	-	HT N	1		
S21	-	-	-	-	-	LT N	1		
S22	+	-	-	-	-	HT A			1

Box Group	OA	P	Sd	Sf	IAA	Phenotype	A (nº)	G (nº)	Sa (nº)
MORAIS									
M01	-	-	-	-	-	LT N		1	
	-	-	-	-	+	LT H		1	
	+	-	+	-	-	LT AS		1	
M02	+	-	-	-	-	LT A	1		
M03	-	-	-	-	-	LT N			2
	+	-	-	-	-	LT A			1
M04	-	-	-	-	-	LT N	6	8	2
	+	-	-	-	-	LT A	2		1
	-	-	+	-	-	LT S	1	1	
	-	-	-	-	+	LT H	8	1	
	+	+	-	-	-	LT AP	1		
	+	-	-	-	+	LT AH	1		
	-	-	+	-	+	LT SH		1	
	-	-	-	-	-	HT N	2	1	1
	-	-	+	-	-	HT S		1	
	-	-	-	-	+	HT H	1		
	+	-	-	-	+	HT AH			1
M05	+	-	-	-	-	LT A			1
	+	-	-	-	+	LT AH	1		
M06	-	-	-	-	-	LT N	1		
	-	-	-	-	-	HT N			1

Box Group	OA	P	Sd	Sf	IAA	Phenotype	A (nº)	G (nº)	Sa (nº)
M07	-	-	-	-	-	LT N		3	
	+	-	-	-	-	LT A			1
	-	-	+	-	-	LT S		1	
	-	-	+	-	+	LT SH		1	
	-	-	-	-	-	HT N	2		
	+	-	-	-	-	HT A			2
M08	-	-	-	-	-	LT N	1	4	1
	+	-	-	-	-	LT A	1	2	5
	-	-	+	-	-	LT S		2	
	-	-	-	-	+	LT H	1	2	
	-	-	+	-	+	LT SH		2	
	-	-	-	-	-	HT N	1	1	4
	+	-	-	-	-	HT A			1
	-	-	-	-	+	HT H	1		1
	+	-	-	-	+	HT AH			2
	+	+	-	-	+	HT APH	1		
M09	+	-	-	-	-	LT A			1
	-	-	-	-	+	LT H		1	
	-	-	-	+	-	HT T			1
	-	-	-	-	+	HT H	1		
M10	-	-	-	-	+	HT H	1		
M11	+	-	-	-	-	LT A	1		1
	-	-	-	-	+	LT H	1	2	
	-	-	-	-	-	HT N	1		
	+	-	-	-	-	HT A		1	
	-	-	-	-	+	HT H	1		
	+	-	-	-	+	HT AH			1
M12	-	-	-	-	-	LT N	1		
	+	-	-	-	-	LT A		1	2
	-	-	-	-	+	LT H	2		
	+	-	-	-	+	LT AH			1
	-	-	-	-	-	HT N	2	1	2
M13	+	-	-	-	-	LT A			1
	-	-	-	-	-	HT N	1		
M14	-	-	-	-	-	LT N			1
M15	+	-	-	-	-	LT A	1		1
	+	-	-	-	+	LT AH	1		
	+	-	-	-	+	HT AH	1		
M16	+	-	-	-	+	HT AH			1
M17	-	+	-	-	-	LT P			1
M18	+	-	-	-	-	LT A			1
M19	-	-	-	-	-	LT N	1		
M20	-	+	-	-	-	LT P	1		
	+	-	-	-	+	LT AH			1
	+	-	-	-	-	HT A			1
M21	-	-	-	-	-	LT N		1	
	-	-	-	-	+	LT H	1		
M22	-	-	-	-	-	HT N			1
M23	-	-	-	-	-	HT N	1		
M24	-	-	-	-	-	HT N	1		

(continued)

Box Group	OA	P	Sd	Sf	IAA	Phenotype	A (nº)
S. BERMEJA							
SB01	-	-	-	-	-	LT N	1
	-	-	+	-	-	LT S	1
	-	-	-	-	-	HT N	1
SB02	+	-	+	-	-	LT AS	1
SB03	-	-	-	-	-	LT N	1
	-	-	-	-	-	HT N	1
SB04	+	-	-	-	-	LT A	3
	-	-	-	-	+	LT H	1
	-	-	-	-	-	HT N	5
	-	+	-	-	-	HT P	1
SB05	+	+	-	-	-	LT AP	1
SB06	-	-	-	-	-	LT N	12
	+	-	-	-	-	LT A	2
	-	+	-	-	-	LT P	1
	-	-	+	-	-	LT S	1
	-	-	-	-	+	LT H	1
	+	-	+	-	+	LT ASH	2
	-	-	-	-	-	HT N	5
	+	-	-	-	-	HT A	1
	-	+	-	-	-	HT P	1
	-	-	-	+	-	HT T	1
SB07	-	-	+	-	-	LT S	1
SB08	-	-	-	-	-	LT N	2
	+	-	-	-	-	LT A	3
	+	-	+	-	-	LT AS	2
	+	-	-	-	+	LT AH	2
	-	-	-	-	-	HT N	3
	-	+	-	-	-	HT P	1
SB09	-	-	-	-	-	LT N	10
	+	-	-	-	-	LT A	2
	-	+	-	-	-	LT P	3
	-	-	+	-	-	LT S	1
	+	-	+	-	-	LT AS	1
	+	-	-	-	+	LT AH	1
	-	+	+	-	+	LT PSH	1
	-	-	-	-	-	HT N	6
	-	+	-	-	-	HT P	1
SB10	-	-	-	-	-	LT N	1
	-	-	+	-	-	LT S	1
SB11	-	-	-	-	+	LT H	1
SB12	+	-	+	-	-	LT AS	1
SB13	+	-	-	-	-	LT A	1
SB14	-	-	-	-	-	LT N	1
SB15	+	-	+	-	-	LT AS	1
SB16	+	-	-	-	-	HT A	1
SB17	-	-	-	-	-	HT N	1
SB18	-	-	-	-	-	LT N	1

Box Group	OA	P	Sd	Sf	IAA	Phenotype	A (nº)
S. NEVADA							
SN01	-	-	-	-	-	LT N	3
	+	-	-	-	-	LT A	2
	-	-	+	-	-	LT S	1
SN02	-	-	-	-	-	LT N	1
SN03	-	-	-	-	-	LT N	1
	+	-	-	-	-	LT A	1
SN04	+	-	+	-	-	LT AS	1
SN05	-	-	-	-	-	LT N	4
	+	-	-	-	-	LT A	3
	-	+	-	-	-	LT P	1
	-	-	+	-	-	LT S	1
	+	-	+	-	-	LT AS	2
	+	-	-	-	+	LT AH	1
	-	-	+	-	-	HT S	1
SN06	-	+	-	-	-	LT P	1
	-	-	+	-	-	LT S	1
SN07	-	-	-	-	-	LT N	5
	-	-	+	-	-	LT S	1
	-	-	-	-	+	LT H	1
	+	-	-	-	+	LT AH	1
	+	-	+	-	+	LT ASH	1
SN08	-	-	+	-	-	LT S	1
SN10	-	-	-	-	-	LT N	2
	+	-	-	-	-	LT A	1
	-	-	+	-	-	LT S	1
	-	-	+	-	+	LT SH	2
SN11	-	-	+	-	-	LT S	1

LT (low Ni tolerance ≤ 2.5 mM Ni MTC), HT (high Ni tolerance 5-10 mM Ni MTC).N: no PGP traits; A: organic acid-producers; P: P-solubilisers; S: Siderophore-producers; T: biosurfactants-producers; H: indoleacetic acid-producers

Table 3.5. BOX-PCR groups and phenotypic characteristics of rhizobacterial strains isolated from the Ni-hyperaccumulating subspecies of *Alyssum*. OA, organic acid-producer; P, phosphate solubilisation capacity; Sf, biosurfactant-producer; Sd, siderophore-producer; IAA, indoleacetic acid-producer. BOX PCR groups were based on profiles obtained for 280 bacterial strains (72 from LAR, 53 from, 61 from SAR and 94 from SBAR). The number of strains for each phenotype and population are given for each BOX group.

Box Group	OA	P	Sd	Sf	IAA	Phenotype	L (nº)	S (nº)	M (nº)	SB (nº)
A01	-	-	-	-	-	LT N				1
	-	+	-	-	-	LT P			1	
A02	+	-	+	-	-	LT AS				1
A03	-	-	-	-	-	LT N		1		
A04	-	-	-	-	-	LT N			1	
A05	+	-	-	-	-	HT A				1
A06	-	-	-	-	-	HT N				1
A07	-	-	-	-	+	LT H			1	
	-	-	-	-	-	HT N	1			
A08	-	-	-	-	-	LT N		1		
	+	-	-	-	-	LT A				1
	+	-	+	-	-	LT AS		1		
	+	-	-	-	+	LT AH			2	
	+	-	-	-	+	HT AH			1	
A09	+	-	-	-	+	LT AH	1			
	-	-	-	-	+	HT H	1			
A10	-	-	-	-	-	HT N			1	
A11	+	-	-	-	-	HT A	1			
A12	-	-	-	-	-	HT N		1		
A13	-	-	-	-	-	HT N		1		
A14	-	-	-	-	-	LT N		1		
	+	-	-	-	-	LT A			1	
	-	-	-	-	+	LT H			1	
	+	-	+	-	-	LT AS				1
	-	-	-	-	-	HT N			1	
	-	-	-	-	+	HT H			1	
A15	+	-	-	-	-	HT A		1		
A16	-	-	-	-	-	LT N			1	
	-	-	+	-	-	LT S				1
	-	-	-	-	+	LT H	1	1	1	
	-	-	-	-	-	HT N	1		1	2
	+	-	-	-	-	HT A	1			
	+	-	+	-	-	HT AS		1		
A17	-	-	-	-	-	LT N	4	2	2	
	+	-	-	-	-	LT A	1			3
	-	-	+	-	-	LT S	1	1		
	-	-	-	-	+	LT H	2		2	
	+	-	-	-	+	LT AH	1			
	-	-	-	-	-	HT N				5
	+	-	-	-	-	HT A		1		
	-	-	+	-	-	HT S	1			
	-	-	-	-	+	HT H	1			
	+	-	-	-	+	HT AH	1			

Box Group	OA	P	Sd	Sf	IAA	Phenotype	L (nº)	S (nº)	M (nº)	SB (nº)
A18	-	-	-	-	+	LT H	1			
	-	+	-	-	+	LT PH		1		
	-	-	-	-	-	HT N			1	
A19	-	+	-	-	+	LT PH		1		
A20	+	-	-	-	-	LT A		1	1	
	-	-	-	-	+	LT H		1	1	
A21	+	-	-	-	+	LT AH			1	
A22	+	-	-	-	-	LT A		1		
	+	+	-	-	-	LT AP				1
	-	-	+	-	+	HT SH	1			
A23	-	-	-	-	-	LT N		1		
	-	+	-	-	-	LT P		1		
A24	-	-	-	-	-	HT N			1	
A25	-	-	-	-	-	LT N		1		
A26	+	-	+	-	-	LT AS				1
	-	-	-	-	+	HT H			1	
A27	+	-	-	-	-	HT A	1			
	-	-	-	-	+	HT H			1	
A28	-	-	-	-	-	LT N				1
A29	-	-	-	-	-	LT N	1			1
	+	-	-	-	-	LT A		1	1	
	-	-	+	-	-	LT S				1
	-	-	-	-	+	LT H				1
A30	-	-	-	-	-	LT N	1			
A31	-	-	-	-	-	LT N			1	1
	+	-	-	-	-	LT A				2
	-	-	-	-	+	LT H	1		1	
	+	-	+	-	-	LT AS		2		
	-	+	+	-	+	LT PSH		1		
	-	-	-	-	-	HT N	1		1	
A32	-	-	-	-	-	LT N			1	1
	+	-	-	-	-	LT A				1
	-	-	-	-	+	LT H			3	
	+	-	+	-	-	LT AS				2
	+	-	-	-	+	LT AH	1			2
	-	-	-	-	-	HT N				2
	-	+	-	-	-	HT P				1
A33	-	-	-	-	-	HT N	2			1
A34	-	-	-	-	-	LT N				1
A35	-	-	-	-	-	LT N	4		1	
	+	-	-	-	-	LT A	2	1	1	1
	-	+	-	-	-	LT P		1		
	-	-	+	-	-	LT S			1	
	-	-	-	-	+	LT H	2	2	1	1
	+	+	-	-	-	LT AP			1	
	+	-	+	-	-	LT AS	1	1		
	+	-	-	-	+	LT AH	1			
	-	-	+	-	+	LT SH		1		
	-	-	-	-	-	HT N			2	
	-	+	-	-	-	HT P				1
	-	-	-	-	+	HT H			1	
	+	-	-	-	+	HT AH	1			

(continued)

Box Group	OA	P	Sd	Sf	IAA	Phenotype	L (nº)	S (nº)	M (nº)	SB (nº)
	-	-	-	-	-	LT N	8	5	2	13
	+	-	-	-	-	LT A	1	3		1
	-	+	-	-	-	LT P				1
	-	-	+	-	-	LT S				2
	-	-	-	-	+	LT H	2		1	1
	+	-	+	-	-	LT AS		3		
	+	-	-	-	+	LT AH	2	3	1	
A36	+	-	+	-	+	LT ASH				2
	-	-	+	-	+	LT SH		1		
	-	-	-	-	-	HT N	3			6
	+	-	-	-	-	HT A	1			1
	-	+	-	-	-	HT P				1
	-	-	-	+	-	HT T				1
	+	+	-	-	+	HT APH			1	
	+	-	+	-	+	HT ASH		1		
	-	-	-	-	-	LT N	6	5	1	10
	+	-	-	-	-	LT A		1	1	2
	-	+	-	-	-	LT P		1		3
	-	-	+	-	-	LT S				1
	-	-	-	-	+	LT H	1	1		
	+	-	+	-	-	LT AS				1
A37	+	-	-	-	+	LT AH				1
	-	+	+	-	+	LT PSH				1
	-	-	-	-	-	HT N	5	3	2	5
	+	-	-	-	-	HT A		1		
	-	+	-	-	-	HT P				1
	-	-	+	-	-	HT S	1	1		
	-	-	-	-	+	HT H			1	
A38	-	-	-	-	-	LT N		1		
	-	-	-	-	+	LT H			1	
A39	-	-	-	-	-	HT N			1	
A40	-	-	-	-	+	LT H	1			

LT (low Ni tolerance ≤ 2.5 mM Ni MTC), HT (high Ni tolerance 5-10 mM Ni MTC).N: no PGP traits; A: organic acid-producers; P: P-solubilisers; S: Siderophore-producers; T: biosurfactants-producers; H: indoleacetic acid-producers.

BOX-PCR profiles of strains associated with the three *Alyssum* subspecies (from L, M, S, SB and SN) were allocated into 40 groups (Table 3.5). Approximately half of these (19 groups) were made up of strains from various populations. On the other hand, some groups were predominantly represented by isolates from a single population. This was the case for groups A36 or A37 which were principally composed of strains isolated from the SB population of *A. malacitanum*, or for groups A08, A14, A20, A23 and A38 which were made up of strains almost exclusively isolated from the Portuguese serpentine populations (M or S) of *A. pintodasilvae*. BOX-PCR group A18 represents isolates associated with *A.*

pintodasilvae from both the Spanish population (L) and Portuguese populations (M and S) (Table 3.5). In general no correlation was found between BOX- and phenotypic-groups, however some phenotypes were shown to be specific to a particular BOX-group. Phenotype SH was specific to the L05 BOX-group in Barazón (Table 3.4), phenotype APH was specific to group M08 in Morais and to S06 in Samil (Table 3.4), and finally, phenotype ASH was specific to group S05 in Samil (Table 3.4). In SB, phenotype PSH was associated with SB09 and in SN phenotype ASH was specific to SN07 (Table 3.4). Regarding the Ni-hyperaccumulators, only phenotype ASH (organic acid-, siderophore- and IAA-producer) was found exclusively in the BOX group A36 (Table 3.5).

Table 3.6. (a) Total numbers of BOX-PCR groups obtained for each site and the number associated with each plant species. (b) Total number of BOX-PCR groups obtained for all the Ni-hyperaccumulators and the number associated with each population.

(a)

	Number of BOX-PCR groups		
Site (total no. of BOX profiles)	**Total**	**Host plant**	
L (n= 119)	19	A	13
		G	13
S (n= 144)	22	A	19
		G	4
		Sa	9
M (n= 139)	24	A	17
		G	8
		Sa	17
SB (n= 94)	18	A	18
SN (n= 42)	10	A	18

(b)

	Number of BOX-PCR groups		
Plant type (total no. of BOX profiles)	**Total**	**Site**	
Ni-hyperaccumulator (n= 280)	40	L	17
		S	20
		M	22
		SB	19

L, Barazón; S, Samil; M, Morais; SB, Sierra Bermeja; SN, Sierra Nevada
A, *Alyssum*; G, *Dactylis*; Sa, *Santolina*

The Shannon diversity index (H´) is presented in Table 3.7. Diversity indices were calculated separately for isolates presenting low (LT) and high (HT) Ni tolerance. No correlation was found between plant species and bacterial diversity

of isolates with low Ni tolerance (LT), the highest H´ values were recorded in the bacterial community associated with the M population of *S. semidentata.* (H´= 1.04) and the S population of *A. pintodasilvae* (H´= 0.95). However, bacterial diversity of isolates with high Ni tolerance (HT), tended to be higher in the rhizosphere of the Ni-hyperaccumulating species than the non-accumulators. The highest index was recorded in the bacterial population of *A. pintodasilvae* growing in Morais (H´= 0.96) while that of the excluder *D. glomerata* growing at the same site presented the lowest bacterial diversity (H´=0.58).

Table 3.7. Shannon diversity index ($H = -\sum(x_i/x_0)\ln(x_i/x_0)$) based on either 16s-rDNA sequences where x_i is the number of isolates of different genera, and x_0 is the total number of strains, or on BOX-PCR profiles where x_i is the number of isolates of different BOX-PCR group (determined according to 82% similarity) belonging to each of the Ni tolerance groups (LT-low tolerance or HT-high tolerance), and x_0 is the total number of strains. L (Barazón), S (Samil), M (Morais), SB (S. Bermeja), SN (S. Nevada).

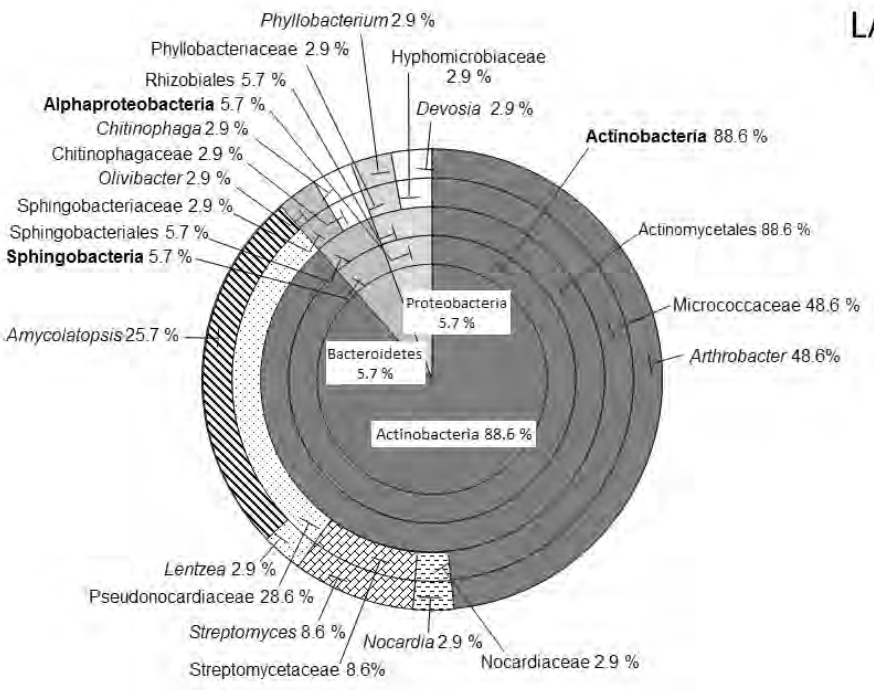
			Shannon index		
			BOX-PCR		16s-rDNA
			LT	HT	
Ni-hyperaccumulators		L	0.78	0.87	1.52
	A. pintodasilvae	S	0.95	0.67	1.33
		M	0.75	0.96	1.74
	A. malacitanum	SB	0.88	0.77	1.53
Non-hyperaccumulators		L	0.82	0.72	1.33
	D. glomerata	S	0.24	0.18	1.14
		M	0.72	0.58	1.25
	S. semidentada	S	0.75	0.58	0.71
		M	1.04	0.85	1.43
	A. serpyllifolium	SN	0.12	-	1.92

16S rDNA identification

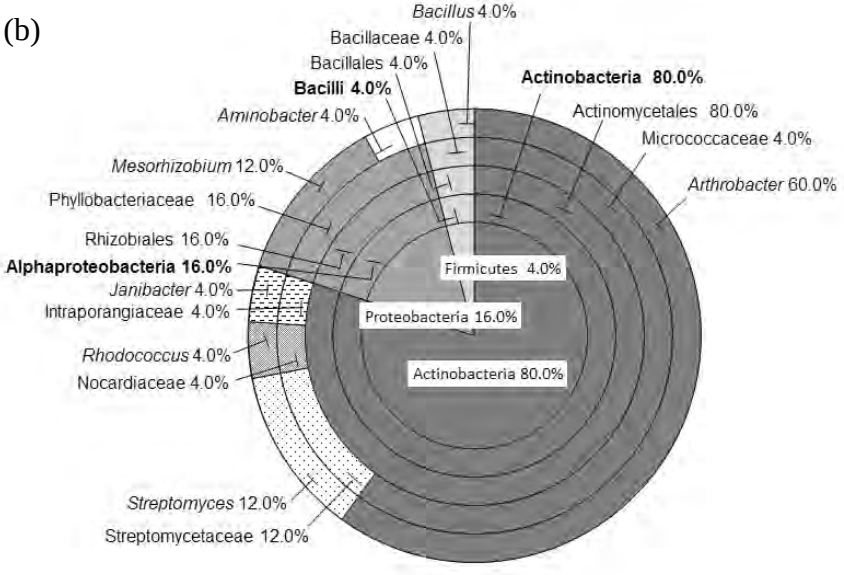
A total of 329 isolates were identified through comparative sequence analysis of 16S rDNA sequences, and isolates were affiliated with a total of 24 different genera belonging to four phyla. The Shannon diversity (H') index was also calculated on the basis of the 16S rDNA sequencing. The highest value was found in the SN population of *A. serpyllifolium* (1.9), while for the serpentine populations, the highest diversity was associated with the Ni-hyperaccumulators, and particularly the S population of *A.pintodasilvae* (1.7) (Table 3.7).

Figure 3.2 presents the taxonomic breakdown for each plant species and population. Four different phyla were represented in the bacterial collection, two

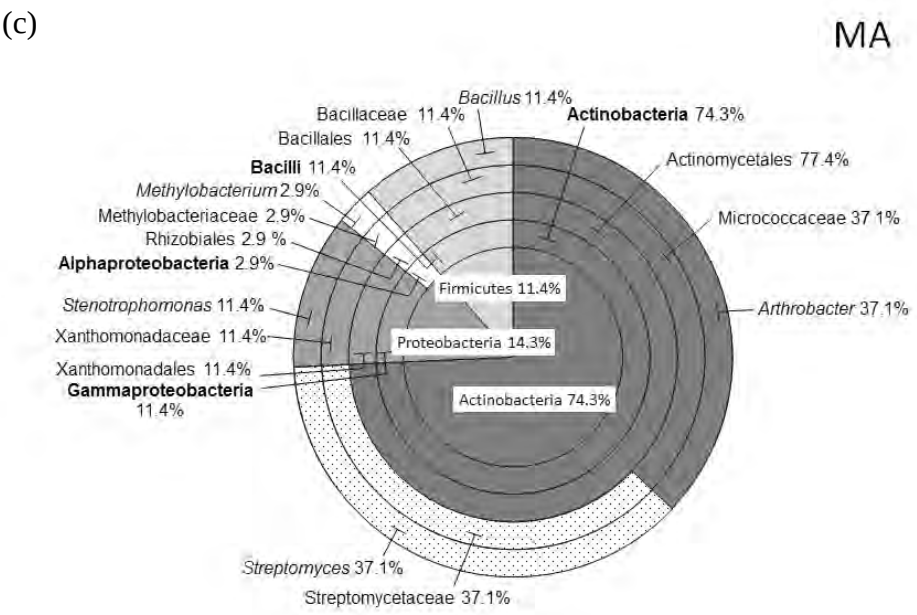
(a)


LA
Phyllobacterium 2.9 %
Phyllobacteriaceae 2.9 %
Hyphomicrobiaceae 2.9 %
Rhizobiales 5.7 %
Alphaproteobacteria 5.7 %
Devosia 2.9 %
Chitinophaga 2.9 %
Actinobacteria 88.6 %
Chitinophagaceae 2.9 %
Olivibacter 2.9 %
Sphingobacteriaceae 2.9 %
Actinomycetales 88.6 %
Sphingobacteriales 5.7 %
Sphingobacteria 5.7 %
Proteobacteria 5.7 %
Micrococcaceae 48.6 %
Amycolatopsis 25.7 %
Bacteroidetes 5.7 %
Arthrobacter 48.6%
Actinobacteria 88.6 %
Lentzea 2.9 %
Pseudonocardiaceae 28.6 %
Streptomyces 8.6 %
Nocardia 2.9 %
Nocardiaceae 2.9 %
Streptomycetaceae 8.6%

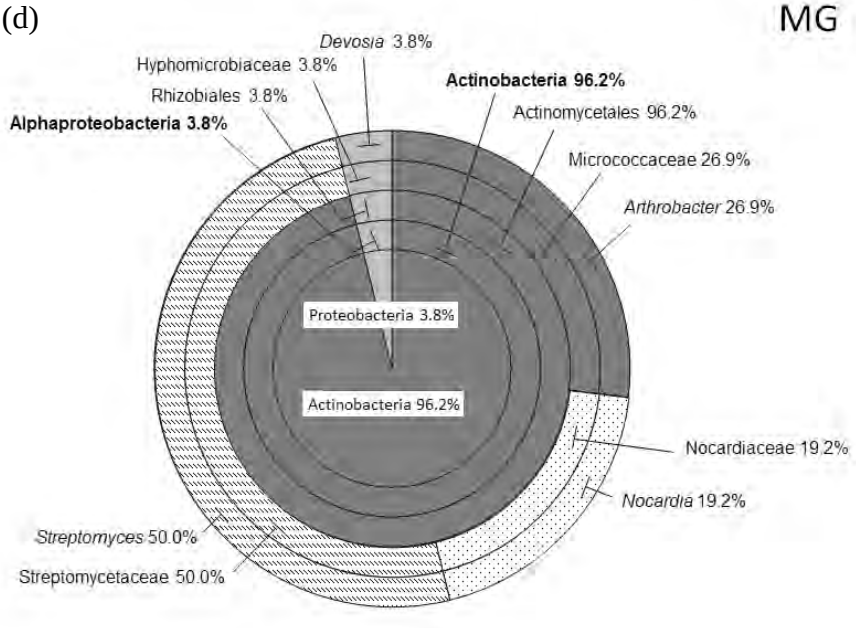
(b)


LG
Bacillus 4.0%
Bacillaceae 4.0%
Bacillales 4.0%
Actinobacteria 80.0%
Bacilli 4.0%
Aminobacter 4.0%
Actinomycetales 80.0%
Micrococcaceae 4.0%
Mesorhizobium 12.0%
Arthrobacter 60.0%
Phyllobacteriaceae 16.0%
Rhizobiales 16.0%
Firmicutes 4.0%
Alphaproteobacteria 16.0%
Janibacter 4.0%
Intraporangiaceae 4.0%
Proteobacteria 16.0%
Rhodococcus 4.0%
Actinobacteria 80.0%
Nocardiaceae 4.0%
Streptomyces 12.0%
Streptomycetaceae 12.0%

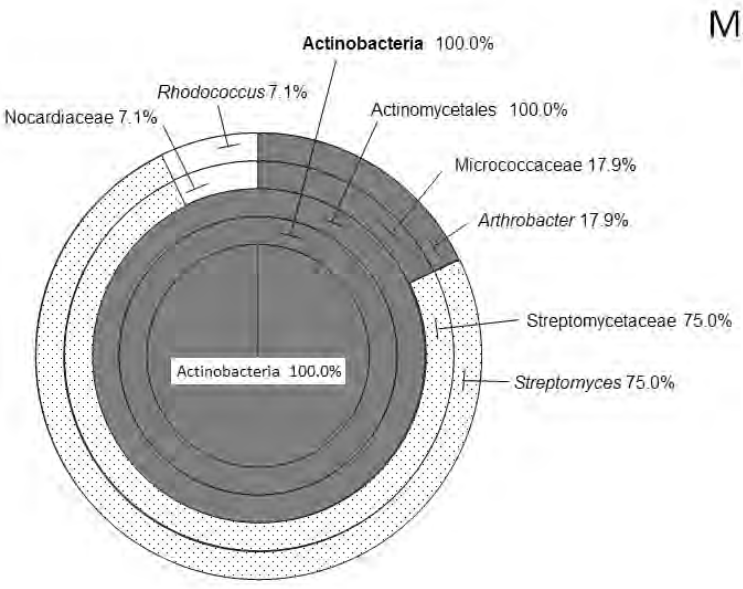
(c)


MA
Bacillus 11.4%
Bacillaceae 11.4%
Bacillales 11.4%
Bacilli 11.4%
Methylobacterium 2.9%
Methylobacteriaceae 2.9%
Rhizobiales 2.9 %
Alphaproteobacteria 2.9%
Stenotrophomonas 11.4%
Xanthomonadaceae 11.4%
Xanthomonadales 11.4%
Gammaproteobacteria 11.4%
Actinobacteria 74.3%
Actinomycetales 77.4%
Micrococcaceae 37.1%
Arthrobacter 37.1%
Firmicutes 11.4%
Proteobacteria 14.3%
Actinobacteria 74.3%
Streptomyces 37.1%
Streptomycetaceae 37.1%

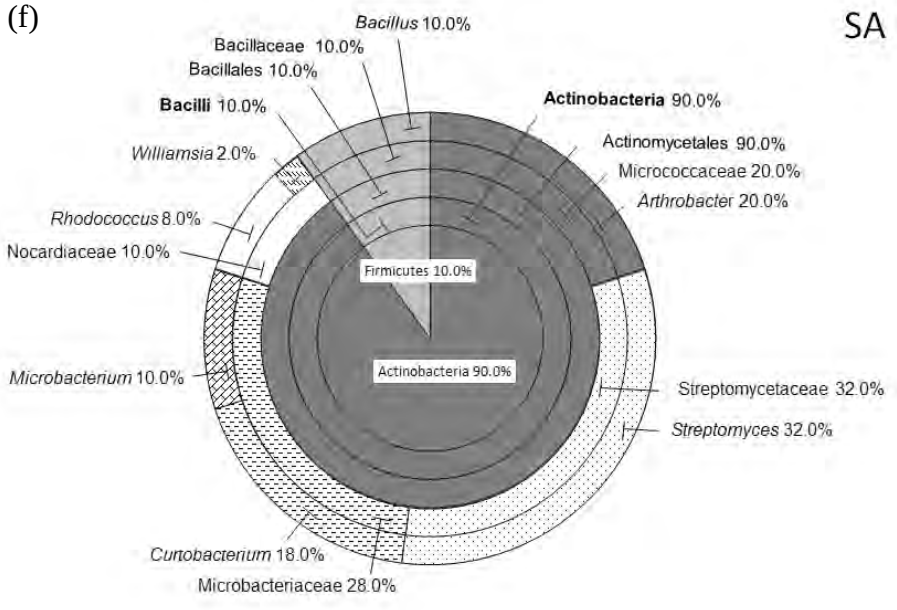
(d)


MG
Devosia 3.8%
Hyphomicrobiaceae 3.8%
Rhizobiales 3.8%
Alphaproteobacteria 3.8%
Actinobacteria 96.2%
Actinomycetales 96.2%
Micrococcaceae 26.9%
Arthrobacter 26.9%
Proteobacteria 3.8%
Actinobacteria 96.2%
Nocardiaceae 19.2%
Nocardia 19.2%
Streptomyces 50.0%
Streptomycetaceae 50.0%

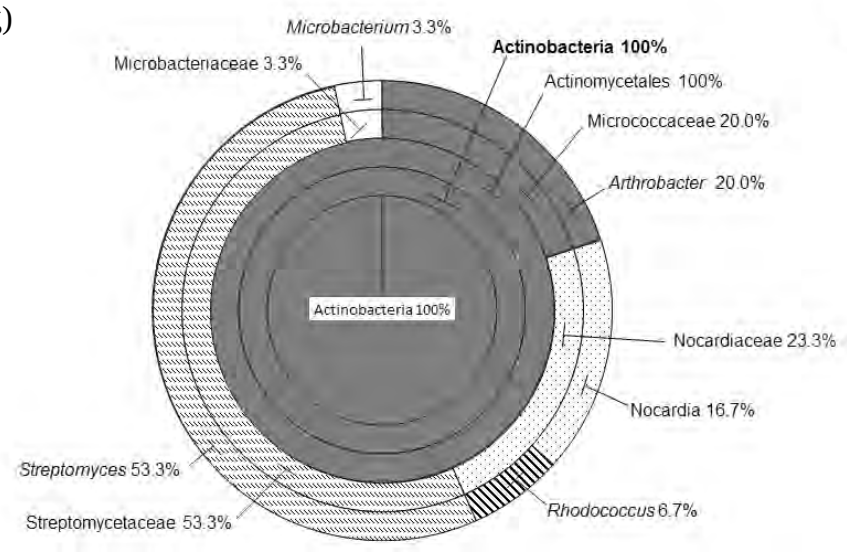
(e)


MS
Actinobacteria 100.0%
Rhodococcus 7.1%
Nocardiaceae 7.1%
Actinomycetales 100.0%
Micrococcaceae 17.9%
Arthrobacter 17.9%
Streptomycetaceae 75.0%
Streptomyces 75.0%
Actinobacteria 100.0%

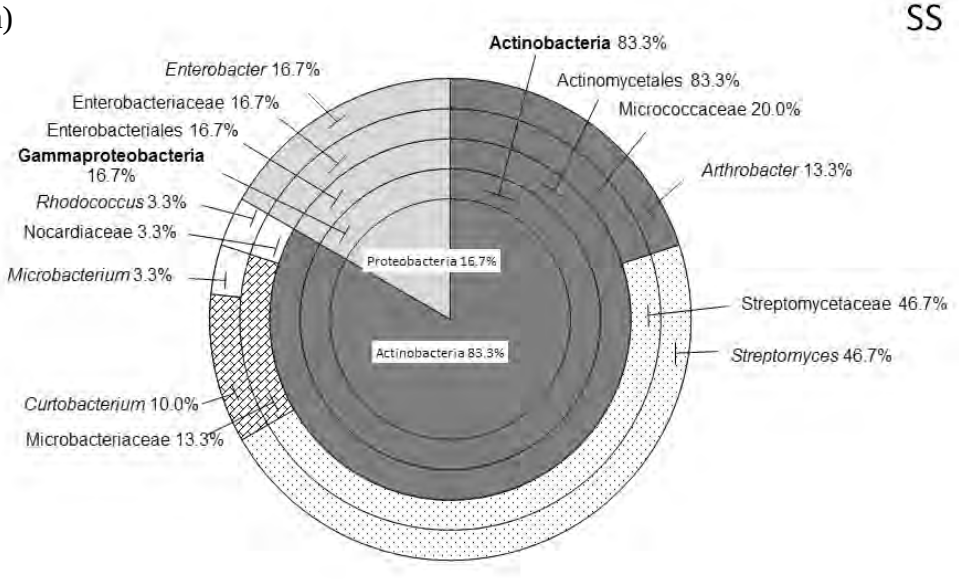
(f)


SA
Bacillus 10.0%
Bacillaceae 10.0%
Bacillales 10.0%
Bacilli 10.0%
Williamsia 2.0%
Rhodococcus 8.0%
Nocardiaceae 10.0%
Microbacterium 10.0%
Actinobacteria 90.0%
Actinomycetales 90.0%
Micrococcaceae 20.0%
Arthrobacter 20.0%
Firmicutes 10.0%
Actinobacteria 90.0%
Streptomycetaceae 32.0%
Streptomyces 32.0%
Curtobacterium 18.0%
Microbacteriaceae 28.0%

(g)


SG
Microbacterium 3.3%
Actinobacteria 100%
Microbacteriaceae 3.3%
Actinomycetales 100%
Micrococcaceae 20.0%
Arthrobacter 20.0%
Actinobacteria 100%
Nocardiaceae 23.3%
Nocardia 16.7%
Streptomyces 53.3%
Rhodococcus 6.7%
Streptomycetaceae 53.3%

(h)


SS
Actinobacteria 83.3%
Enterobacter 16.7%
Actinomycetales 83.3%
Enterobacteriaceae 16.7%
Micrococcaceae 20.0%
Enterobacteriales 16.7%
Gammaproteobacteria 16.7%
Arthrobacter 13.3%
Rhodococcus 3.3%
Nocardiaceae 3.3%
Proteobacteria 16.7%
Microbacterium 3.3%
Streptomycetaceae 46.7%
Actinobacteria 83.3%
Streptomyces 46.7%
Curtobacterium 10.0%
Microbacteriaceae 13.3%

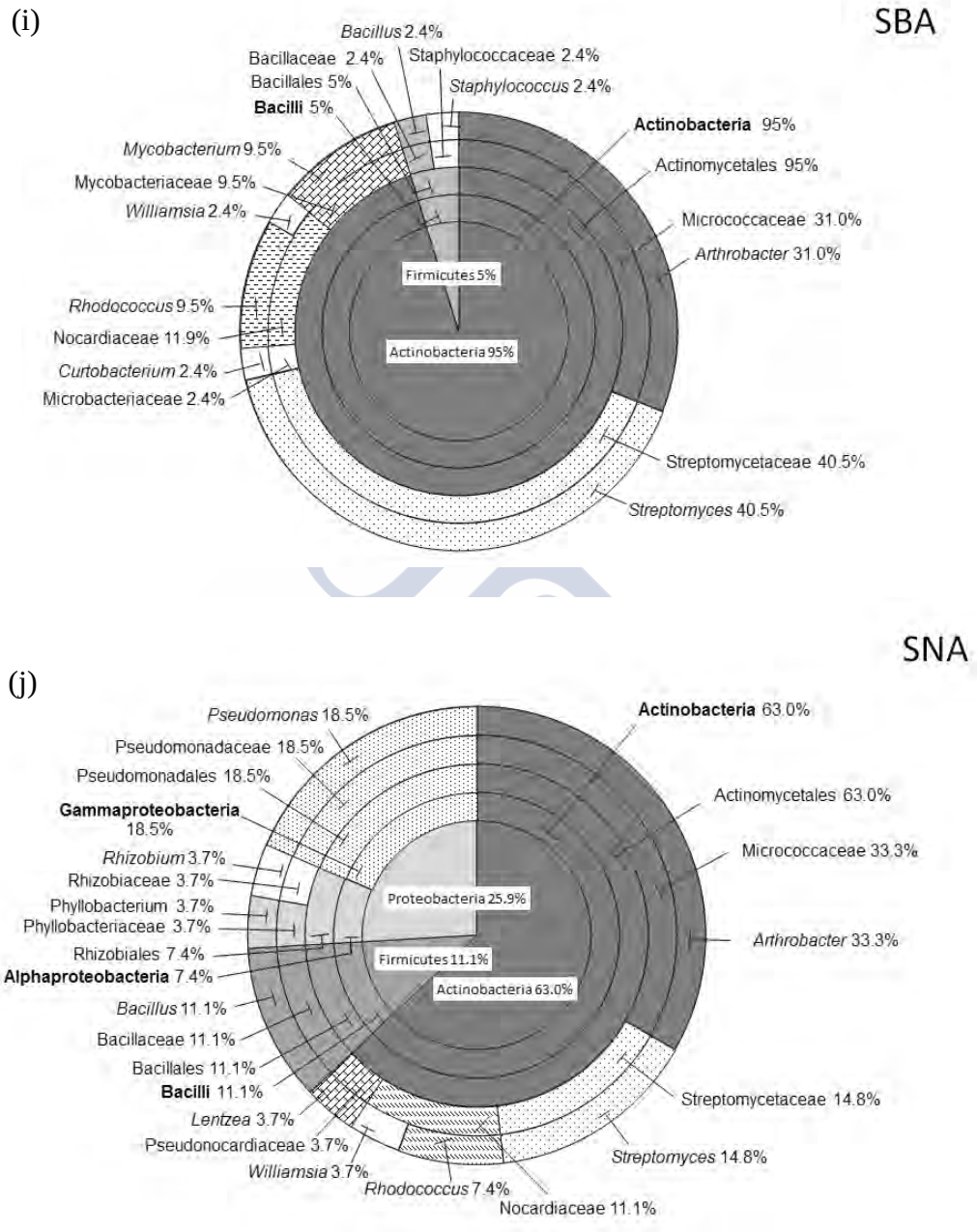


Figure 3.2. Taxonomic breakdown of 16s rDNA sequences (a) 35 sequences from *Alyssum pintodasilvae* (Barazón), (b) 25 from *Dactylis glomerata* (Barazón), (c) 35 from *Alyssum pintodasilvae* (Morais), (d) 26 from *Dactylis glomerata* (Morais), (e) 28 from *Santolina semidentata* (Morais), (f) 50 from *Alyssum pintodasilvae* (Samil), (g) 30 from *Dactylis glomerata* (Samil), (h) 30 from *Santolina semidentata* (Samil), (i) 42 from *Alyssum malacitanum* (S. Bermeja), (j) 26 from *Alyssum serpyllifolium* (S. Nevada). The central pie shows percentages by phyla; each outer ring progressively breaks these down to finer taxonomic levels (phyla, class, family, genera).

phyla correspond with Gram-positive bacteria (*Actinobacteria* and *Firmicutes*) and two with Gram-negative (*Bacteroidetes* and *Proteobacteria*). The distribution of isolates within these phyla differed according to the soil type, 94% of the isolates obtained from serpentine soils (L, S, M, SB) were Gram-positive bacteria and 6% were Gram-negative (data not shown). In contrast, in the calcareous soil (SN) Gram-positive bacteria represented 77% of the total community and Gram-negative 23%. *Proteobacteria* or *Actinobacteria* were the most diverse taxonomic classes (including 9 and 11 different genera, respectively).

Differences in bacterial diversity were observed regarding the distribution of phyla between plant species and populations. For the M population of *S. semidentata* (MS) and the S population of *D. glomerata* (SG) 100% of the isolates were affiliated with the phylum *Actinobacteria* (Figure 3.2e and Figure 3.2g). For the Ni-hyperaccumulators, the proportion of isolates belonging to the *Actinobacteria* decreased in the order SB (95%) (Figure 3.2i), S (90%) (Figure 3.2f), L (88.6%) (Figure 3.2a) and M (74.3%) (Figure 3.2c). *Actinobacteria* was least represented in the SN population of *A. serpyllifolium*, although 63% of isolates were still affiliated with this phylum (Figure 3.2j). The second most important phylum was *Proteobacteria* (*Alpha-* and *Gamma-proteobacteria*), particularly in the SN population of *A. serpyllifolium* (25.9% of isolates belonged to this phylum) (Figure 3.2j). The phylum *Firmicutes* was primarily associated with the *Alyssum* subspecies (11.4% of isolates in M, 10.0% in S, 5% in SB and 11.1% in SN) (Figure 3.2c, Figure 3.2f, Figure 3.2i and Figure 3.2j), while isolates belonging to the Bacteroidetes were only found in the L population of *A. pintodasilvae* (5.7% of isolates) (Figure 3.2a).

Regarding the distribution of genera amongst the bacterial isolates associated with each plant species (considering all their populations together), the Ni-hyperaccumulators showed a higher diversity and host members of 19 different genera, while only 9 genera were associated with the non-hyperaccumulating *A. serpyllifolium*. The rhizosphere community of *D. glomerata* and *S. semidentata* were represented by members of 10 and 6 genera, respectively (data not shown). Some bacterial genera, such as *Arthrobacter*, *Streptomyces* and *Rhodococcus* (all members of the *Actinobacteria*), were found in the rhizosphere of all the plant species (Figure 3.2a to Figure 3.2j).

In contrast, some genera were only isolated in specific plant species: members of the genera *Stenotrophomonas* (*Gammaproteobacteria*), *Methylobacterium* (*Alphaproteobacteria*), *Staphylococcus* (*Firmicutes*), *Amycolatopsis* and

Mycobacterium (both *Actinobacteria*) and the phylum *Bacteroidetes* (exclusively represented by the genera *Olivibacter* and *Chitinophaga*) were only found in association with the Ni-hyperaccumulators (either *A. pintodasilvae* or *A. malacitanum*) (Figure 3.2a, Figure 3.2c, Figure 3.2f and Figure 3.2i). On the other hand, members of the genera *Janibacter* (*Actinobacteria*) and *Mesorhizobium* and *Aminobacter* (*Alphaproteobacteria*) were exclusively isolated from the rhizosphere of *D. glomerata* (specifically its L population, Figure 3.2b), and *Enterobacter* (*Gammaproteobacteria*) from *S. semidentata* (S population, Figure 3.2h). Finally, members of two genera, *Pseudomonas* and *Rhizobium* (*Gamma-* and *Alpha-proteobacteria*, respectively), were only found in the rhizosphere of the non-hyperaccumulator *A. serpyllifolium* (Figure 3.2j).

Amongst the Ni hyperaccumulators the predominant genera represented by isolates associated with the L population of *A. pintodasivae* (Figure 3.2a) were *Arthrobacter* (48.6%) and *Amycolatopsis* (25.7%). While the main genera associated with the two Portuguese populations of *A. pintodasilvae* were (Figure 3.2c and Figure 3.2f): *Arthrobacter* (37.1% in M and 20% in S) and *Streptomyces* (37.1% in M and 32.0% in S). Similarly, the rhizosphere community of *A. malacitanum* (SB) (Figure 3.2i) was predominantly represented by *Arthrobacter* (31.0%) and *Streptomyces* (40.5%). The latter two genera were also important components of the rhizobacterial community of the non-hyperaccumulator *A. serpyllifolium*, but in this case 18.5% of isolates were identified as *Pseudomonas*. As observed for the hyperaccumulators, members of the genera *Arthrobacter* and *Streptomyces* were predominant in the rhizosphere of both *D. glomerata* and *S. semidentata*: 60% of isolates of the L population of *D. glomerata* were affiliated with *Arthrobacter*, while 53% and 75% of isolates from S and M populations of *D. glomerata* and *S. semidentata*, respectively, were affiliated with *Streptomyces*. Members of the genera *Nocardia* were generally found in association with *D. glomerata* from Portuguese sites (M and S), representing 19.2% and 16.7% of isolates, respectively.

3.4. Discussion

This study describes the culturable rhizosphere bacterial community associated with different populations of the only two Ni-hyperaccumulating subspecies of *Alyssum serpyllifolium* in the Iberian Peninsula. The Ni tolerance, PGP traits and diversity of these communities were compared with those of non-hyperaccumulating plants growing at the same sites and with a close relative and

non-hyperaccumulator, *A. serpyllifolium*, growing in calcareous soils. Trace metals are well known to affect the soil bacterial density and activity, as well as the community structure and diversity (Bååth, 1989). A high soil metal content has been suggested to explain the lower bacterial densities observed in serpentine soils compared to nearby non-serpentine soils (Amir and Pineau, 1998; Pal *et al.*, 2005). Higher bacterial densities in the non-serpentine (SN) soil were therefore to be expected. Toxic concentrations of trace metals were not detected in this calcareous soil and the higher organic matter content should favour microbial development (Acea and Carballas, 1986). In accordance, bacteria from the serpentine soils (L, M, S, SB) showed a higher resistance to Ni than those from the calcareous (SN) soil. An increase in the Ni concentration of the culture medium led to a significant decrease in bacterial densities of all soils. However, this decrease was less pronounced in the serpentine soils, indicating their adaptation to the phytotoxic levels of Ni to which they are normally exposed. Microorganisms from serpentine soils have previously been shown to present a high level of tolerance to trace metals, such as Ni, Co and Cr (Schlegel *et al.*, 1991; Pal *et al.*, 2005; Becerra-Castro *et al.*, 2009; Turgay *et al.*, 2012).

Plant root exudates, secretions and lysates contain labile C sources and growth factors which are well known to stimulate microbial growth and metabolic activity (Grayston *et al.*, 1998; Delorme *et al.*, 2001). Bacterial densities were therefore unsurprisingly higher in the rhizosphere soils of all the plant species studied compared to corresponding non-vegetated soils (R / NV ratios > 1). This rhizosphere effect was most pronounced in the case of *Alyssum* (in both the Ni-hyperaccumulators and non-hyperaccumulator). The Ni-hyperaccumulating *A. pintodasilvae* and *A. malacitanum* also hosted a higher density of Ni-resistant bacteria in their rhizosphere than either the non-accumulating plants from serpentine sites or non-vegetated soil. This effect was not observed in the case of the non-hyperaccumulator *A. serpyllifolium* (SN). Population-specific differences were observed in the case of *A. pintodasilvae*, and the highest densities of Ni-resistant bacteria were associated with the M population. In addition, both the M population of *A. pintodasilvae* and the SB population of *A. malacitanum* showed a selective enrichment of Ni-resistant bacteria in the rhizosphere. This has been observed for other metal-hyperaccumulating plants, such as *Alyssum bertolonii*, *Noccaea goesingensis* and *Sebertia acuminata* (Schlegel *et al.*, 1991; Mengoni *et al.*, 2001; Lodewyckx *et al.*, 2002; Mengoni *et al.*, 2010). Likewise, Zn tolerance has often been described in bacterial communities exposed to this metal: Delorme *et al.*

(2001) found higher densities of Zn-tolerant bacteria in the rhizosphere of the Zn-hyperaccumulator *N. caerulescens* compared to the non-accumulator *Trifolium pratense* or to non-vegetated soil. Moreover, bacteria isolated from serpentine soils have been shown to present co-resistance to various metals, including Cr, Co and Zn (Mengoni *et al.*, 2010). It would also have been interesting to study root architecture systems of the different plant species and in particular to compare those of the different populations of *Alyssum*. Root architecture and proliferation would influence exudation and therefore also the rhizosphere effect.

Trace metal uptake by hyperaccumulators has been found to be associated with partial depletion of labile, easily bioaccessible trace metal pools in the rhizosphere (e.g. Whiting *et al.* (2001); Fitz *et al.* (2003); Puschenreiter *et al.* (2005)) and active root proliferation towards soil patches rich in metals (Schwartz *et al.*, 1999). Such depletion of labile metal pools has often been associated with sustained or even enhanced metal solubility (i.e. soil solution concentration) due to a more intense weathering of Ni-bearing silicates in the rhizosphere (Puschenreiter *et al.*, 2005; Kidd *et al.*, 2009). In agreement, water-soluble Ni concentrations in the rhizosphere soils of the Ni-hyperaccumulating *Alyssum* from M and SB were significantly higher than those detected in the rhizosphere soil of non-accumulators (e.g. *D. glomerata* or *S. semidentata*) or of non-vegetated soil. In addition, previous studies have also shown a higher labile Ni fraction (assessed via sequential extraction procedures or $Sr(NO_3)_2$-extractable concentrations) in the rhizosphere soil of these *Alyssum* populations compared to non-vegetated soils or non-accumulators (Becerra-Castro, 2006; Cabello-Conejo, 2015). This tendency towards a higher Ni mobility in the rhizosphere of the hyperaccumulator could be attributed to the activity of the Ni-tolerant bacteria and at the same time lead to their further enrichment in the rhizosphere. Of the 508 isolates obtained from serpentine soils the vast majority (65%) were able to growth with the presence of Ni in the medium (with concentrations > 1mM Ni). In contrast, 90% of isolates from the close relative of the Ni-hyperaccumulator, *A. serpyllifolium* were affected by the presence of the minimal concentration of Ni tested (1 mM) and none of them were able to tolerate more than 2.5 mM. Our results are in agreement with previous studies: Abou-Shanab *et al.* (2003) found the majority of rhizobacteria isolated from serpentine soils in Oregon (USA) were able to grow in the presence of 8 mM Ni, and Mengoni *et al.* (2001) found bacterial isolates from serpentine soils in Tuscany (Italy) resisted between 7 and 10 mM Ni in their growth medium. These authors did not study the Ni resistance of bacterial isolates associated with

non-accumulators growing at the same site. Here the MTC for isolates associated with the hyperaccumulators was higher than that of the Ni-excluder *D. glomerata*, which could reinforce the idea that the activity of these hyperaccumulator plants leads to an increase in labile Ni and hence enrichment in Ni-tolerant bacteria or that this plant group selects for Ni-tolerant bacteria which in turn modify soil Ni availability. However, Ni-resistance was an equally important phenotype of the bacterial isolates associated with the serpentinophyte *S. semidentata* (a similar % of isolates showed Ni MTC of 10 mM Ni), indicating that this may not be a phenomenon specific to the hyperaccumulators.

The potential application of these Ni-tolerant plant-associated bacteria in phytoextraction (or phytomining) processes is based on their ability to improve plant growth and biomass production and/or modify soil metal availability and plant uptake. Bacterial strains for bioaugmentation trials are frequently selected on the presence of PGP traits (such as the production of IAA, ACC deaminase or siderophore production) or their ability to release compounds which could potentially modify metal bioavailability (such as the production of biosurfactants, siderophores or organic acids) (Abou-Shanab *et al.*, 2003; Idris *et al.*, 2004; Braud *et al.*, 2006; Cabello-Conejo *et al.*, 2014). Half of the isolates obtained in this study showed at least one PGP trait (54% of isolates). The rarest phenotypes were the biosurfactant-producers and the ability to solubilise inorganic phosphate. Biosurfactant-producing strains were only detected amongst isolates from *A. malacitanum*, *D. glomerata* or *S. semidentata* (and were identified as members of the genus *Bacillus* (*Firmicutes*) and *Arthrobacter* (*Actinobacteria*)). Some authors have shown that microbial produced surfactants can increase the mobility of Cd, Cu, Pb and Zn in soil, and in some cases, increase plant metal uptake (Venkatesh and Vedaraman, 2012). It would be interesting to further characterise the biosurfactant-producing bacterial strains identified in this study and particularly their Ni-solubilising capacities. Isolates which were able to solubilise inorganic phosphate were primarily associated with the Ni-hyperaccumulators (at most 12% isolates). Most of these were *Actinobacteria* and identified as members of the genera *Arthrobacter* (strains SA37, SA40, SBA82, SNA110), *Streptomyces* (strains SA46, SBA57, SBA89) or *Rhodococcus* (strains SBA86 and SBA70). One P-solubilising isolate from the M population of *A. pintodasilvae* was identified as *Methylobacterium* (*Alphaproteobacteria*). Phosphate-solubilising bacteria are effective in promoting plant growth and biomass by releasing P from inorganic and organic P pools through solubilisation and mineralisation (Rodríguez and

Fraga, 1999). These bacterial associations may play an important role in P mobilisation for hyperaccumulating species of *Alyssum*, which seems to form non or weak mycorrhizal associations since reports of arbuscular mycorrhizal symbiosis with hyperaccumulating *Alyssum* sp. cannot be found in the literature.

Jeong *et al.* (2013) inoculated *Brassica juncea* with strains of phosphate-solubilising *Bacillus* sp. According to these authors the release of organic acids and drop in soil pH led to a mobilisation of Cd and consequent increase in Cd uptake as well as an enhanced plant biomass. An increase in plant growth and P uptake were reported by Ma *et al.* (2010) after inoculating *Ricinus communis* and *Helianthus annuus* with the rhizobacterial strain *Psychrobacter* sp. SRS8. Since metal-enriched soils are characteristically deficient in essential nutrients such as P, the identification of bacterial inoculants which could potentially improve the nutritive state of the phytoextracting plant are of interest. More than 50% of the hyperacccumulator associated P-solubilisers also showed Ni tolerance (tolerating Ni concentrations in the growth medium of $\geq$ 2.5 mM). One of these strains (*Arthrobacter nicotinovorans* SA40) was shown to significantly improve the biomass production of *A. pintodasilvae* when grown in contrasting Ni-rich soils (serpentine soils and sewage sludge-amended agricultural soils) (Cabello-Conejo *et al.,* 2014). Strain SA40 was also characterised as a siderophore- and IAA-producer. The fact that this strain has a beneficial effect on plant growth in both naturally-rich and anthropogenic-contaminated soils is noteworthy since it could be applied to a wider range of soils with contrasting edaphic properties.

A substantial number of isolates were able to produce organic acids, siderophores or IAA, although the frequency of these traits varied according to the host plant species or population. Plant-associated bacteria have been shown to modify soil trace metal availability through the release of organic acids, such as citric, oxalic or acetic acid, which in turn can lead to an increase in plant metal uptake (Sessitsch *et al.,* 2013). More than 50% of organic acid-producers isolated from the hyperaccumulators or *S. semidentata* tolerated > 2.5 mM Ni and some of them showed additional PGP characteristics. In a previous study, Becerra-Castro *et al.* (2011) showed that by using the cell-free culture of 13 of the strains associated with the *Alyssum* sp. (included in this study) the extractable Ni concentration from serpentine soils was increased. However, we found no relation between the capacity for Ni mobilisation and the phenotypic traits of the strains. In a more detailed study, strain LA44 (identified as *Arthrobacter nitroguajacolicus*) was shown to be an efficient mobiliser of Ni from ultramafic rocks under *in vitro*

conditions, and principally liberated Ni associated with Mn oxides through the exudation of oxalate (Becerra-Castro *et al.*, 2013). This strain also shows intense IAA-production, is highly Ni-resistant, and in a previous study was able to increase shoot Ni concentrations in *A. pintodasilvae* growing in serpentine soil (likely due to an enhanced Ni phytoavailability and hence plant uptake). The strains identified in this study as P-solubilisers or organic acid-producers, and particularly those which show high Ni tolerance, would be good candidates for further studies related to Ni mobilisation.

Zaidi *et al.* (2006) reported that an IAA-producing *Bacillus subtilis* strain was able to promote the growth of *Brassica juncea* and thereby increased Ni extraction. In this study, IAA-producers were particularly associated with the Ni-hyperaccumulator *A. pintodasilvae*, while the production of siderophores was associated with strains isolated from *D. glomerata*. The fact that siderophore-producing bacteria were found in association with the grass species is interesting since this type of plant is known to be a highly efficient phytosiderophore producer. The presence of associated siderophore-producing bacteria could be related to competition between the bacteria and the plant for obtaining Fe. Siderophore-producing bacterial strains were mainly affiliated with the genera *Streptomyces* and *Arthrobacter*, although one isolate associated with *D. glomerata* was identified as *Microbacterium* sp. (strain SG22) and one isolate associated with *A. malacitanum* was identified as *Mycobacterium* sp. (strain SBA60). Abou-Shanab *et al.* (2003) found that inoculating ultramafic soils with the actinobacterial *Microbacterium arabinogalactanolyticum* AY509224 increased soil Ni extractability and uptake by *Alyssum murale*. The predominance of siderophore-producers within the isolate collection obtained from the non-hyperaccumulator (*A. serpyllifolium*) could be associated with the limited availability of Fe in calcareous soils (due to the pH-dependent low solubility of Fe). The appearance of a specific phenotype linked to a particular plant species also suggests a plant-driving effect in microorganism selection, while specific phenotypes associated with sites indicates that the soil conditions are important factors shaping the bacterial community.

Bacterial diversity was assessed using the BOX-PCR technique and 16S rDNA sequencing. Although the majority of BOX-PCR groups were affiliated with isolates from two or three different plant species, or from different populations of the same species, some were specific to a certain plant population or plant species. The phenotypes associated with the different BOX-PCR groups, shows

that there is no correlation between BOX-patterns and phenotype, probably because of the high intra-BOX phenotypic variability. These results are in agreements with Mengoni *et al.* (2001), who found no correlation between OTUs obtained by ARDRA (restriction analysis of 16S) and heavy-metal tolerant phenotype in bacterial strains isolated from the rhizosphere of *A. bertolonii*.

To our knowledge differences in the bacterial communities associated with different populations of the same Ni-hyperaccumulating species have not previously been shown. The phyla *Proteobacteria* and, in particular, *Actinobacteria*, dominated the culturable rhizobacterial community. Isolates were affiliated with members of genera, such as *Arthrobacter, Streptomyces, Rhodococcus* or *Microbacterium*, which have been frequently described amongst soil bacteria. Bacteria of the phyla *Proteobacteria* (particularly *Alphaproteobacteria*) and *Actinobacteria* were also the most numerous within the rhizosphere of the Ni-hyperaccumulator *Alyssum murale* (Abou-Shanab *et al.*, 2010). In the rhizosphere of *Alyssum bertolonii* the culturable bacterial population was dominated by Ni-resistant *Pseudomonas* strains (*Gammaproteobacteria*) (Mengoni *et al.*, 2001). In this study, strains identified as *Pseudomonas* sp. were exclusively found in the rhizosphere of *A. serpyllifolium* growing in the calcareous soil. Culturable rhizobacteria associated with the Ni-hyperaccumulator *N. goesingensis* were mainly represented by *Methylobacterium* spp., an alphaproteobacterial genus, as well as *Rhodococcus* spp. and *Okibacterium* spp., belonging to the *Actinobacteria* (Idris *et al.*, 2004). Gremion *et al.* (2003) constructed clone libraries based on the 16S rRNA and 16S rDNA and found *Actinobacteria* to be a dominant part of the metabolically active bacteria in the rhizosphere of the Cd/Zn-hyperaccumulator *N. caerulescens*. The predominance of actinobacterial strains in serpentine soils has been related to the high adaptability of such gram-positive bacteria to toxic concentrations of trace metals (DeGrood *et al.*, 2005). Metal toxicity or nutrient deficiency has been shown to lower the frequency of r-strategists (bacteria capable of rapid growth and utilization of resources), such as *Bacillus* or *Pseudomonas*, as these are more sensitive to toxic substances (Kozdrój 1995; Kunito *et al.*, 2001). This could partly explain the lower frequency of r-strategists in the serpentine soils. On the other hand, despite a reduced growth and metabolic activity, k-strategists (such as the Actinomycetales) present stable populations over a long time period (making them recommended candidates for bioaugmentation purposes) (Lebeau *et al.*, 2008). The major differences in taxonomic diversity were therefore found between serpentine and non-serpentine soils.

Since the effects of the same bacterial inoculum on plant growth and metal bioaccumulation can be both plant- (Becerra-Castro *et al.,* 2012) and soil-specific (Cabello-Conejo *et al.,* 2014), the selection of microorganisms adapted to the soil characteristics and an ability to colonise the rhizosphere will be a pre-requisite for successful bioaugmentation. This study generated a large number of plant-associated rhizobacterial strains with potential application in these techniques. Many were affiliated with genera which have previously been shown to be beneficial for soil metal removal processes. Although plant population-specific differences in the diversity of associated rhizobacteria were not large and the Ni-hyperaccumulators did not seem to harbour a characteristic bacterial taxonomic diversity; a small number of strains were only found in association with the *Alyssum* hyperaccumulators and would be interesting candidates for further studies related to the application of Ni hyperaccumulating species in phytomining. For example, strains MA98 and MA106 both identified as *Stenotrophomonas* sp. and IAA- and organic acid-producers, respectively; MA91 which is a P-solubiliser (identified as *Methylobacterium* sp.); LA24, LA22, and LA23, all of them IAA-producers (identified as *Amycolatpsis* sp.*)* or SBA60 identified as *Mycobacterium* sp. and siderophore-producers. Moreover, the beneficial effects of stains could also be more pronounced when these are used as a mixed inoculum. These strains will be used in future bioaugmentation trials (individually or as mixed inoculum) to evaluate their effects on the Ni phytoextraction capacity of Ni-hyperaccumulating plant species.

3.5. References

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Chapter 4

Nickel availability in the rhizosphere of the Ni-hyperaccumulator *Alyssum pintodasilvae* assessed via conventional soil analysis and diffusive gradients in thin films (DGT)

ABSTRACT

Soil Ni availability was studied in the rhizosphere of the Ni-hyperaccumulator *Alyssum pintodasilvae* and the Ni-excluder *Holcus lanatus*. Ni availability was evaluated using conventional extractions, whereas Ni fluxes in soil solution and soil replenishment capacity for Ni were assessed using the diffusive gradient in thin films (DGT) technique and DIFS modelling. Both plant species modified their surrounding soil: an increase in dissolved organic C (DOC), cation exchange capacity (CEC), and improvement in the Ca/Mg ratio were observed. Soil Ni availability was higher in the rhizosphere, and there was a shift in Ni fractionation to more plant-available Ni fractions. All these changes were more pronounced in the hyperaccumulator, and the DGT technique was more sensitive to plant-induced changes in element availability than conventional extractions. Modelling the DGT data showed that the rhizosphere soil of *H. lanatus* was able to sustain the lower initial soluble Ni concentration present in this soil through replenishment from the solid phase, while Ni resupply in the rhizosphere soil of the hyperaccumulator was not sufficient to maintain the high initial concentrations of soluble Ni in the soil solution over time.

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4.1. Introduction

The extreme ability of Ni-hyperaccumulators to accumulate Ni in their harvestable tissues makes these plants particularly useful for phytomining strategies (Brooks *et al.*, 1998; Chaney *et al.*, 2007). Phytomining offers the opportunity to recover valuable metals, such as Ni, from metal-enriched biomass of hyperaccumulators. Ni extraction efficiency from this bio-ore has been shown to be high (up to 25% Ni in plant ashes) since these ashes present low impurities (Chaney *et al.*, 2007; Zhang *et al.*, 2014). Moreover, crops of hyperaccumulators can be grown in low-grade ores (usually lower than 3%) which could not be exploited using conventional mining extraction techniques (van der Ent *et al.*, 2015). For phytomining to be economically viable it is necessary to maximise the Ni uptake efficiency of hyperaccumulators and their biomass yield. Agronomic practises such as bioaugmentation with beneficial microorganisms or soil fertilisation regimes have been shown to significantly increase biomass production of metal hyperaccumulators (Abou-Shanab *et al.*, 2006; Cabello-Conejo *et al.*, 2014; Bani *et al.*, 2015). Since metal availability greatly influences the success of phytomining and its long-term sustainability, more in-depth studies of rhizospheric processes determining metal availability and replenishment could help to further improve soil management practises aimed at increasing Ni availability. To date, mostly non-specific rhizosphere mechanisms have been proposed to explain metal uptake by these plants, and several studies have reported that hyperaccumulators access the same metal pools as non-accumulating plants (Hutchinson *et al.*, 2000; Salt *et al.*, 2000; Massoura *et al.*, 2004; Echevarria *et al.*, 2006; Hammer *et al.*, 2006). Massoura *et al.* (2004) studied an excluder plant species (*Triticum aestivum*), an indicator species (*Trifolium pratense*) and three populations of the Ni-hyperaccumulator *A. murale* to determine whether or not the available Ni pool in the soil varied for the different species. Results showed that, for a given soil, the available pools were similar for all three plant species and that they all accessed the same Ni exchangeable pool regardless of their Ni uptake capacity. However, studies such as that of Fitz *et al.* (2003) found sustained As solubility in soil when growing the As-hyperaccumulator *Pteris vitatta*, and at the same time a decrease in the labile As pool. These authors suggested that the capacity and velocity of the soil to supply metals to the soil solution from less available pools could be a critical factor determining the total amount of metal available for plant uptake.

The labile metal concentration estimated through single extractions is

often poorly correlated with plant metal uptake, making it difficult to elucidate the effects of hyperaccumulators on soil metal availability; since, as suggested previously, soil metal bioavailability is dependent on the concentration in the soil solution but also on the rate of transport through the soil (Hooda and Zhang, 2008). Diffusional Gradient in Thin Films (DGT) was originally developed by Davison and Zhang (1994) to concentrate and measure trace elements in natural waters, but since then, has been increasingly used to estimate the availability of nutrients and/or inorganic contaminants in soils and sediments. DGT is a passive sampling technique where a polyacrylamide gel with an element binding agent mimics plant root uptake. Solutes diffuse from the soil and are adsorbed onto the resin causing depletion in the soil solution concentration in contact with the DGT. This depletion induces a resupply of ions from dissociation of complexes and release from the solid phase (Ernstberger *et al.*, 2005; Lehto *et al.*, 2006a).

Metal availability determined by DGT has been shown to correlate better with plant metal uptake than other estimations of available metal fractions (Degryse *et al.*, 2009; Puschenreiter *et al.*, 2013). Moreover, DGT also measures the extent of the depletion caused by the resin gel through the *R* ratio: C_{DGT} / Csol, where C_{DGT} is the time averaged concentration of metals at the interface of the DGT device, and Csol is the concentration of metals in the soil solution before DGT deployment (Harper *et al.*, 1998; Ernstberger *et al.*, 2005). The R ratio is affected by the solid-phase labile pool size and the response time of the soil to the depletion, and therefore gives an indication of soil resupply capacity. DGT measurements can be modelled using the 2D DIFS (DGT Induced Fluxes in Sediments) software (Sochaczewski *et al.*, 2007). DIFS measures the dependence of *R* on two main parameters: K_D, which is the distribution ratio of metals between the solid phase and the soil solution, and T_C, which is the rate of exchange between both pools. Chemical imaging of labile metals can be done using DGT coupled with laser-ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) (Kreuzeder *et al.*, 2013; Santner *et al.*, 2015). High-resolution imaging of DGT-measured metal fluxes showed increased fluxes of Cd and Zn around the roots of the Cd/Zn-accumulator *Salix smithiana* (Hoefer *et al.*, 2015). However, this technique has not yet been used to assess Ni fluxes in Ni-hyperaccumulating plant species.

The aim of this work was to compare plant-induced effects on Ni availability and fractionation between the Ni-hyperaccumulator *A. serpyllifolium* subsp. *lusitanicum* Dudley and P. Silva (endemic to the Iberian Peninsula) and the

Ni-excluder *Holcus lanatus* L. Both these plant species can be found growing at the same serpentine outcrop. The Ni availability of non-vegetated and rhizosphere soils was assessed using conventional extractants and the DGT technique. Ni fractionation was estimated using a sequential extraction procedure, while Ni kinetics and the soil replenishment capacity were assessed through the C_{DGT}/Csol ratio and DIFS modelling. DGT was also applied to both plant species growing in serpentine soil in rhizotrons for the chemical imaging of labile Ni distribution along the root axis.

4.2. Materials and Methods

Study soil and plant species

Plants and non-vegetated soil (NV) were collected in the serpentinitic area of Barazón (NW Spain), which belongs to one of the main alloctonous ophiolitic units in the Iberian Peninsula. The site is included in the Serra do Careón and covers an area of approximately 65 km^2 which is considered a Special Area of Conservation (SAC) and is included within the Natura 2000 Network. Ultramafic rocks in the area are characterised by a high degree of serpentinization, but in scarcely foliated and less serpentinized rocks a harzburgitic composition can be inferred: olivine, orthopyroxene, tremolitic amphibole, chlorite, brown spinel and magnetite. Amphibole and chlorite are retrograde products after orthopyroxene (Díaz García *et al.*, 1999). The soils show abundant rocky otucrops with limited edaphic evolution, and the most frequent soils are Mollic Leptosols and Haplic Phaeozems (IUSS, 2015). Barazón has a European humid-temperate climate with a mean annual temperature of 12.9ºC and mean annual precipitation of 1381 mm (Carballeira *et al.*, 1983). The whole plant and root system (including the root ball) of five to six individuals of the Ni-hyperaccumulator *Alyssum serpyllifolium* subsp. *lusitanicum* Dudley and P. Silva (hereafter referred to as *A. pintodasilvae*) and the non-accumulator *Holcus lanatus* L. were collected. The Ni-hyperaccumulator is endemic to the serpentine outcrops of NW Spain and NE Portugal. Rhizosphere soil was operationally defined as the soil attached to roots after gentle crushing of the root ball and shaking the root system. Tightly held soil (< 3 mm from the root surface) was considered rhizosphere soil. To the extent possible, root debris included in the collected rhizosphere soil were removed using tweezers or by sieving. Moreover, five surface soil samples (0-15 cm) were collected at each site from bare patches where no plants were found growing (NV).

Plant and soil analysis

Plant material was separated into shoots and roots, washed with pressured tap water followed by deionised water, oven-dried at 45ºC, weighed and ground. Plant tissues (approximately 0.1 g) were digested in a 2:1 concentrated NHO_3:HCl mixture on a hot plate at 160ºC. The Ca, Mg, K, P, Fe, Co, Cu, Mn and Ni concentrations were measured by ICP-OES (Vista Pro; Varian Inc., Australia). Data were expressed on the basis of dry weight of plant material. NV and rhizosphere soils were sieved through a 2-mm stainless steel sieve. For each plant species, the rhizosphere soil samples were pooled together and thoroughly mixed to form one single composite sample. Soil samples were kept at 5ºC until their analysis. Soil pH was measured in H_2O and 0.1 M KCl (1:2.5 w/v). Total C and N were analysed by combustion with a CHN analyser (Model CHN-1000, LECO Corp., St Joseph, MI). Soils were digested in a 3:1 mixture of concentrated HNO_3:HCl and Ni concentrations were determined by ICP-OES. Available P was determined by the Olsen's $NaHCO_3$ method (Olsen *et al.*, 1954). Cation exchange capacity (CEC) was estimated using 1 M NH_4Cl as the sum of the exchangeable cations Ca, Mg, Al, Na and K. Dissolved organic C (DOC) was determined in 1:2.5 (w/v) soil:H_2O extracts after 2 h of shaking (adapted fromJones and Willett (2006)). Samples were centrifuged at 3750 *g* for ten minutes, filtered at 0.22 µm and acidified with HNO_3 (70%). DOC concentration was determined using a Vario TOC Cube (Elementar Analysensysteme GmbH, Hanau, Germany).

A metal fractionation scheme was carried out for Ni according to the method of Zeien and Brümmer, (1989). First, soil samples were shaken with 1 M NH_4NO_3 (unbuffered) for 24 h at room temperature, and the solution was separated by centrifugation (15 min; 3750 x *g*), filtered, and stabilised with HNO_3 (14.4 N). This extracts the water-soluble and exchangeable metals (fraction 1 [F1]). Second, the resulting residue was shaken for 24 h at room temperature with 1 M ammonium acetate (NH_4OAc; adjusted with 50% acetic acid [HOAc] to pH 6), centrifuged, filtered, and stabilised with HNO_3 (14.4 N). NH_4NO_3 (25 mL) was added to the remaining residue and shaken for 10 min. The solution was separated by centrifugation, filtered, and combined with the NH_4OAc extract. This step extracts easily mobilisable metals (F2). Third, the residue was shaken with 0.1 M $NH_2OH.HCl$ plus 1 M NH_4OAc (adjusted to pH 6 with diluted HCl) for 30 min, separated by centrifugation, filtered, and stabilised with HCl (12 N). NH_4OAc (25 mL; 1 M) was added to the residue and shaken for 10 min, centrifuged, filtered (repeated once), and combined with the above-mentioned solution. This extracts

metals bound to Mn oxides (F3). Fourth the residue was shaken with 50 mL 0.025 M NH_4-EDTA (adjusted to pH 4.6 with diluted NH_4OH) was added to the residue, and after shaking over-head for 90 min the solution was separated by centrifugation (15 min, 3750 x *g*) and filtered. A second volume of 25 mL 1M NH_4OAc (adjusted to pH 4.6 by 100% HOAc) was added. After shaking overhead for 10 min, the solution was separated by centrifugation, filtered and combined with the NH_4EDTA extract. This extracts metals bound to organic matter (F4). Fifth, 50 mL 0.2 M NH_4-oxalate (equivalent to 0.2 M diammonium oxalate monohydrate plus 0.2M oxalic acid dihydrate adjusted to pH 3.3 with diluted NH_4OH) was added to the residue, and after shaking (overhead) for 4 h in the dark, the solution was separated by centrifugation (15 min; 3750 x *g*) and filtered. A second volume of 25 mL 0.2 M NH_4-oxalate (pH 3.3) was added to the residue and shaken overhead in the dark for 10 min. The solution was separated by centrifugation (15 min; 3750 x *g*) and filtered, and the two filtrates were combined. This step targets those metals bound to amorphous Fe oxides (F5). Sixth, 50 mL 0.1 M ascorbic acid plus 0.2 M NH_4-oxalate [equivalent to 0.1 M L(+)-ascorbic acid plus 0.2 M NH_4-oxalate buffer-0.2M oxalic acid dihydrate adjusted to pH 3.25 with diluted NH_4OH] were added, and after shaking horizontally in a water bath for 30 min at 96°C ±3°C, the solution was separated by centrifugation (15 min; 3750 x *g*) and filtered. The remaining solution was extracted with 25 mL 0.2 M NH_4-oxalate (pH 3.3) by shaking overhead in the dark for 10 min. The solution was separated by centrifugation (15 min; 3750 x *g*) and filtered, and the filtrates were combined. This extracts metals bound to crystalline Fe oxides (F6). Finally, the remaining residue was digested with aqua regia to determine the residual (silicate-bound) fraction (F7). The Ni concentration was analysed in all the extracts by ICP-OES.

Soil metal availability was assessed though single extractions (10 mM Sr $(NO_3)_2$ (Everhart *et al.*, 2006) and 50 mM $Ca(NO_3)_2$ (McLaren *et al.*, 2007) 1:4 w/v 2 h shaking) and by the passive sampling technique Diffusive Gradients in thin Films (DGT) (detailed in the following section). The Ni concentration was analysed in the soil extracts by ICP-OES.

Diffusive Gradient in Thin Films (DGT) measurements

The small amount of soil available from each rhizosphere necessitated using a reduced amount of soil for the DGT deployments following the approach by Muhammad *et al.* (2012). Soil slurries were prepared by mixing air-dried and

sieved (2 mm mesh) rhizosphere soil with deionised water up to the soils' maximum water holding capacity (MWHC). Sets of replicate slurries from each soil were prepared 48 h before DGT deployments to allow for equilibration. Soil solution was sampled from one set of soil slurries before DGT deployment: slurries were transferred to plastic tubes and centrifuged at 3750 × g for 10 min. The supernatant was removed, filtered using 0.22 µm filter paper, acidified with 1 M HNO_3, and the concentrations of Al, Ca, Co, Fe, Mn and Ni (Csol) were determined by ICP-OES.

DGT samplers were assembled by placing together a 0.4-mm Chelex gel disc, overlain with a 0.8-mm polyacrylamide diffusive gel (DGT Research Ltd., UK) and a 0.45 µm membrane filter (Supor 450, Pall Corporation, New York USA). A constant mass of soil slurry from each soil (NV and rhizosphere) was placed onto the DGT-probe window, ensuring that no bubbles were formed in the slurry or at the sample window. The soils were then left on the DGT devices for four different times (24, 72, 168 and 264 hours) in a water-saturated atmosphere at 25ºC in an air tight-plastic container. Upon retrieval, the DGT devices were washed with deionised water to remove soil particles, dissembled and the resin gels were then eluted with 10 mL of 1M HNO_3 in closed microvials. The concentration of Al, Ca, Co, Fe, Mn and Ni in the eluent was measured by ICP-OES.

The accumulated Ni mass in the chelating resin (M, µg) was calculated as follows (eq 1)

$$M = C_e(V_n + V_{rg})/f_e \qquad \text{(eq 1)}$$

C_e	metal concentration in the extract where the resin gel was eluted (in mg L^{-1})
V_n	volume of 1M HNO_3 where the chelating resin is eluted (10 mL)
V_{rg}	volume of chelating resin (0.18 mL)
f_e	correction factor that compensates for the fraction of metals that cannot be released from the chelating (0.8 for Ni and Mn and 0.7 for Fe)

From the accumulated mass of metal (M) the Flux (F, µg cm^{-2} h^{-1}) and the DGT-labile metal concentration C_{DGT} (µg cm^{-3}) were calculated as follows (eq 2 and eq 3):

$$F = M / At \qquad \text{(eq 2)}$$

M	accumulated mass (µg)
A	exposed gel area (cm^2)
t	deployment time (s)

$$C_{DGT} = (M\,\Delta g/ADt) \qquad \text{(eq 3)}$$

M	accumulated mass (µg)
Δg	diffusion layer thickness (diffusion gel + filter) (cm)
A	exposed gel area (cm^2)
D	diffusion coefficient of metal in the diffusive gel ($cm^2\ s^{-1}$)
T	Deployment time (s)

C_{DGT} is the time-averaged concentration at the DGT interface. This was used to calculate the *R* ratio, which represents the ratio of the mean metal concentration at the DGT surface and the bulk soil solution metal concentration (Csol) (eq 4). *R* is a measure of resupply from the solid phase.

$$R = C_{DGT} / C_{Sol} \qquad \text{(eq 4)}$$

DIFS modelling

The 2D DIFS software (Sochaczewski *et al.*, 2007; Lehto *et al.*, 2008) was used to assess the kinetics of Ni resupply to both plant types. Briefly, the model considers the soil to have two separate pools for trace metals: the solution (*C*, mol cm^{-3}) (where all metal is assumed to be both labile and mobile) and labile solid phase metal (C_S, mol g^{-1}), and that the dynamics of exchange between these two pools are governed by first order kinetics, which are described by the rate constants k_f and k_b (representing the adsorption and desorption rate constants, respectively) (Sochaczewski *et al.*, 2007; Lehto *et al.*, 2008). K_D ($cm^3\ g^{-1}$) defines the ratio of the sorbed (C_S) to dissolved concentration (*C*) at equilibrium while the response time (T_C) represents the characteristic time for the perturbed system (after deployment of DGT device) to reach 63% of its equilibrium position

(Jannasch *et al.*, 1988; Sochaczewski *et al.*, 2007). The DGT measured ratio *R*, is dependent on the deployment time and the extent of depletion, which is determined by the quantity of metal available for supply from the solid phase (K_D) and the rate of this resupply (T_C). 2D DIFS provides an automated, iterative method that determines K_D and T_C for a series of *R* measurements determined by DGT. Key parameters and input variables of 2D DIFS are described in Table 4.1.

Table 4.1. Key parameters and variables of the 2D DIFS

Parameters		
ϕ_s	sediment layer porosity	
ϕ_d	diffusion layer porosity	
P_C	particle concentration	g cm^{-3}
Diffusion coefficients		
D_S	sediment layer diffusion coefficient	cm^2 s^{-1}
D_d	diffusion layer diffusion coefficient	cm^2 s^{-1}
D_0	water diffusion coefficient	cm^2 s^{-1}
DGT parameters		
Δg	diffusion layer thickness	mm
M	mass in the resin layer	mol cm^{-2}
F	DGT flux	mol cm^{-2} s^{-1}
R	ratio of DGT estimated to pore water concentration	
Variables		
domX, domY	domain sizes (X, Y)	mm
t	time of deployment	s
divT	number of time steps	
Process Parameters		
C	initial dissolved concentration	mol cm^{-3}
C_S	initial sorbed concentration	mol g^{-1}
T_C	sediment response time	s
K_D	distribution ratio	cm^3 g^{-1}
k_f	sorption rate constant	s^{-1}
k_b	desorption rate constant	s^{-1}

Harper *et al.* (2000); Sochaczewski *et al.* (2007) and Lehto *et al.* (2008) describe two extreme cases of soil responses according to their combinations of K_D and T_C. In the sustained case, metal resupply is sufficiently fast to maintain the pore water concentration adjacent to the DGT device close to its initial values (the soil is characterised by a large metal labile phase (K_D) and low response time (Tc)). In the diffusive case, there is no exchange reaction between metal in the sorbed phase and the soil solution and the soil is characterised by a small labile metal pool (low K_D) or a very high response time (high Tc).

K_D and Tc were determined for each soil by comparing the experimentally determined R values from the different length deployments to R values obtained by simulating those deployments while subjecting them to different K_D and T_c values (Ernstberger *et al.*, 2002; Ernstberger *et al.*, 2005). The fit between the modelled and experimentally determined R values was tested visually following the approach by Ernstberger *et al.* (2005). The small set of data points and the small amount of soil used in the deployments allowed for qualitative comparison between the K_D and Tc values from different soils. The sensitivity of the model system was tested by changing the fitted K_D and Tc values by 30%. To express numerically how well the R data fits the estimated K_D and T_C the following error function was used (eq 6).

$$E = 100. \sqrt{\sum_{i=1}^{N} (R_e(t_i) - R_s(t_i))^2} \qquad \text{(eq 6)}$$

Where $R_e(t_i)$ is the experimentally derived R value and $R_s(t_i)$ is the simulated value for time t_i.

Calculated and estimated parameters

-R, M and F were calculated as described above K_D ($cm^3\ g^{-1}$ distribution coefficient): Ratio of the sorbed (estimated using F1 of the fractionation scheme) to dissolved concentration (Cs). The sorbed concentration represents readily exchangeable, rather than total, material associated with the solid phase.

-Pc ($g\ cm^{-3}$ particle concentration): Ratio of the mass of particles to volume of pore water in a given volume of soil, calculated using the porosity (ϕ) and the density of the particulate matter, dp ($g\ cm^{-3}$), using (eq 7):

$$P_c = d_p\,(1-\phi)/\phi \qquad \text{(eq 7)}$$

-ϕ (porosity) was calculated assuming a standard particle density of 2.65 g cm^{-3}.

$$\phi = 1 - d_p \ / 2.65 \qquad \text{(eq 8)}$$

-D_0 (cm^2 s^{-1}, diffusion coefficient in water)

-D_d (cm^2 s^{-1}, diffusion coefficient in the diffusive layer): it has been found experimentally that for the diffusion layer $D_d = D_0 \, (\theta^2 = 1)$ (Davison and Zhang, 1994).

-Ds (cm^2 s^{-1} diffusion coefficient in the soil): it has been found that diffusion coefficients in soils are related to the self-diffusion coefficients (D_0) by tortuosity Θ^2 (eq 9)

-Θ^2: Tortuosity is not used as a parameter in DIFS, instead Ds is specified directly. Boudreau (1997) gives a number of proposed relationships that relate tortuosity to the more readily measured porosity.

$$D_s = D_0 \ / \ \Theta^2 \qquad \text{(eq 9)}$$

High-resolution Ni fluxes along plant roots

The DGT was also used to measure the two-dimensional distribution of Ni fluxes along the root axis of either *A. pintodasilvae* or *H. lanatus*. Plants were grown for two months in rhizotrons filled with Ni-rich serpentine soil (< 2mm) collected at the serpentine outcrop of Barazón. Soils were fertilised with 150 kg N ha^{-1} (in the form of NH_4NO_3) and mixed with 10% of sand (w/w) to reduce compaction before planting. Rhizotrons are flat plastic growth containers where roots develop preferentially along the front plate since plants are grown at an inclination. The rhizotrons used in this study had inner dimensions of 40 x 10 x 1.5 cm (height x width x depth). To prevent the front plate from sticking to soil and plant roots, a 0.05 mm thick polytetrafluorethylene (PTFE) foil was fixed before closing the rhizotrons and soil was saturated to 60% WHC. After trans-planting the plants, the rhizotrons were wrapped in aluminum foil to avoid algae growth and allow roots to develop. The experiment was carried out in a growth room at 30-40% relative humidity (day/night cycle: 16/8h) and soil water content was kept at approx. 60% WHC throughout the experiment through regular additions of water. For chemical imaging, polyacrylamide diffuse gradients in thin film (DGT) gels containing suspended particulate reagent iminodiacetate (SPR-IDA) resin (CETAC, Nebraska, USA) were prepared as described previously (Warnken *et al*., 2004; Hoefer *et al*., 2015; Valentinuzzi *et al*., 2015). DGT gel

strips (approx. 2 x 4 cm) were deployed in the region of interest during 24 h. A 10 μm thick polycarbonate filter membrane (Nucleopore, Whatman, Maidstone, UK) was used as a diffusive layer between the soil and DGT gel. The gels were retrieved, rinsed and dried using a gel dryer and fixed onto glass plates prior to laser ablation inductively-coupled plasma mass spectrometry (LA-ICP-MS) analysis; using a UP 193-FX208 (ESI, NWR Division, CA, US) laser ablation system coupled to a NexION 350D ICP-MS (Perkin-Elmer, MA, USA).

Preparation of standards and gel analysis were carried out as previously described (Hoefer *et al.*, 2015; Valentinuzzi *et al.*, 2015). Resin gel discs were assembled with a diffusion layer and a membrane filter (as for soil analysis) and immersed into well stirred 1 M $NaNO_3$ solutions containing Ni (as $Ni(NO_3)_2$) at concentrations of 6.3, 62.8, 627.6, 1882.8 $\mu g\ L^{-1}$. Two replicates of each standard were prepared, one gel was eluted in 10 mL of 1 M HNO_3 and the solutions were then analysed by ICP-MS. The eluent concentration was used to calculate the mass of metal bound by per unit area of resin gel, and the other gel was dried and analysed by LA-ICP-MS.

4.3. Results

Soil physicochemical properties and plant ionome

Soil physicochemical characteristics were typical of serpentine soils, presenting a close to neutral pH (pH 6.8), low total C and N content (1.34% and 0.13%, respectively), low CEC (6.65 $cmol_c\ kg^{-1}$) principally dominated by Mg (5.74 $cmol_c\ kg^{-1}$), Ca/Mg ratio of 0.15, low available P (4.3 $mg\ kg^{-1}$) and K (22 $mg\ kg^{-1}$), and high pseudo-total Ni concentration (3607 $mg\ kg^{-1}$). The shoot ionome of both plant species is presented in Table 4.2. The Ni-hyperaccumulator presented mean shoot Ni concentrations of 9 $g\ kg^{-1}$, while the Ni-excluder presented a mean shoot Ni concentration of 0.01 $g\ kg^{-1}$. The Ni-hyperaccumulator also presented significantly higher shoot concentrations of macronutrients, such as Ca (up to 40-fold higher), Mg (10-fold), P (5-fold) and K (1.5-fold), than the non-accumulator. Similarly, concentrations of Co, Cu, Fe and Mn were higher in the shoots of *A. pintodasilvae* than *H. lanatus*. The Ca/Mg ratio in the hyperaccumulator reached values of close to 2, while this ratio was consistently <1 in the grass species.

Table 4.2 .Concentrations of macro- and micro-nutrients in the shoots of *Alyssum pintodasilvae* and *Holcus lanatus*.

	A. pintodasilvae	*H. lanatus*
Ca (g kg^{-1})	25.4 ±0.8	0.7 ±0.3
K	9.4 ±1.1	6.5 ±1.0
Mg	11.4 ±0.1	1.1 ±0.1
Ni (mg kg^{-1})	9108 ±893	13 ±3
Co	19.4 ±1.5	0.4 ±0.0
Cu	6.1 ±0.9	4.2 ±0.3
Fe	255 ±36	116 ±20
Mn	232 ±41	52 ±5
P	952 ±131	207 ±40

Soil Ni availability and fractionation

Figure 4.1 shows the properties of the two rhizosphere soils compared to the NV soil. Soil pH was slightly higher in the rhizosphere of the hyperaccumulator (pH 6.9), and significantly lower in the rhizosphere of the excluder (pH 6.5; $p < 0.05$), compared to NV soil. Significantly higher concentrations of DOC were found in rhizosphere soil of both species compared to NV soil. Moreover, DOC was significantly higher in the rhizosphere of *A. pintodasilvae* (67.6 mg kg^{-1}) than of *H. lanatus* (42.7 mg kg^{-1}) ($p < 0.05$). Both CEC and the Ca/Mg ratio were also significantly higher in the rhizosphere of the hyperaccumulator (9.87 $cmol_c$ kg^{-1} and 0.40, respectively) than in either NV soil (6.65 $cmol_c$ kg^{-1} and 0.15) or the rhizosphere of *H. lanatus* (7.70 $cmol_c$ kg^{-1} and 0.17) ($p < 0.05$). On the other hand, available P (P olsen) was slightly but not significantly higher in the rhizosphere of *A. pintodasilve* ($p > 0.05$) than either NV or *H. lanatus*.

Ni availability in NV soils was always lower than in the rhizosphere soils, and this was independent of plant species. Plant species-specific differences were also observed in both $Sr(NO_3)_2$- and $Ca(NO_3)_2$-extractable Ni concentrations, and in C_{DGT} concentrations of Ni (Figure 4.1). For the simple extractions, extractable concentrations of Ni were approximately 2-fold lower in NV soil than in

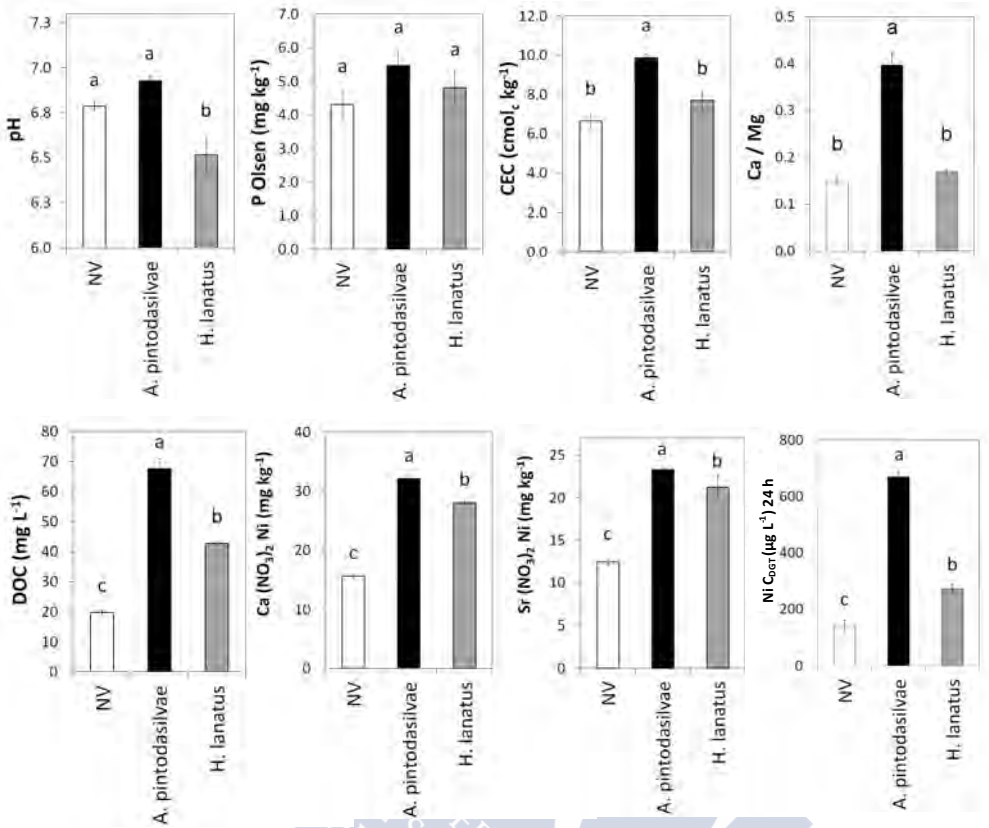


Figure 4.1. Properties of the non–vegetated (NV) and *Alyssum pintodasilvae* and *Holcus lanatus* rhizosphere soils and Ni availability (assessed through single extractions ($Ca(NO_3)_2$ and $Sr(NO_3)_2$) and diffusive gradient in thin films (DGT)). Error bars indicate the standard error of the mean (n = 3). Different letters indicate significant differences between soils ($p \leq 0.05$).

rhizosphere soils. Both $Sr(NO_3)_2$- and $Ca(NO_3)_2$-extractable Ni concentrations were slightly, but significantly, higher in the rhizosphere of the Ni-hyperaccumulator than *H. lanatus*. More pronounced differences were observed using DGT: C_{DGT} concentrations of Ni were between 2- and 5-fold higher in rhizosphere soils than NV soil. Moreover, C_{DGT} concentrations of Ni in the rhizosphere of *A. pintodasilvae* were up to 2.5-fold higher than in the rhizosphere of *H. lanatus*. $Sr(NO_3)_2$- and $Ca(NO_3)_2$-extractable Ni were positively correlated with DOC (R^2 = 0.8-0.9).

Figure 4.2a shows the Ni fractionation in NV and rhizosphere soils. In all cases, the residual (silicate-bound) fraction (F7) was the most important metal pool (representing between 62 and 66% of the total Ni), and this was followed by Ni bound to crystalline Fe oxides (F6) (representing 23 to 24% of total Ni).

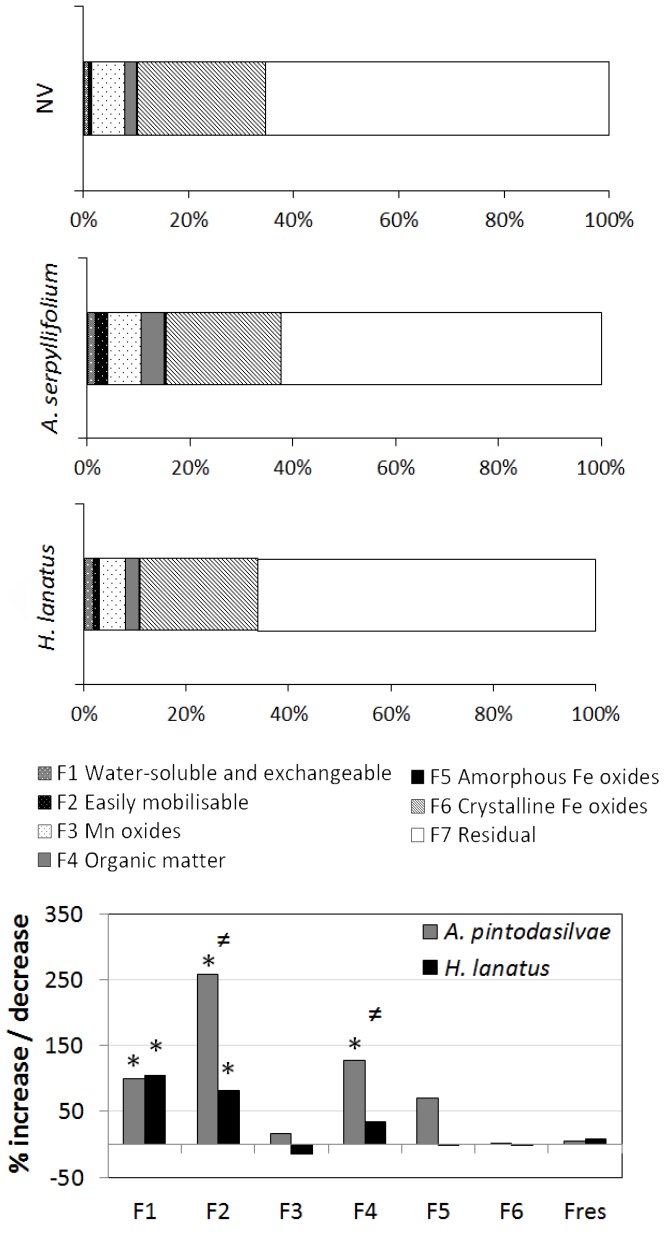


Figure 4.2. Soil Ni fractionation in non-vegetated (NV) and rhizosphere soils and the mean decrease/increase (%) of each fraction in the rhizosphere soils relative to the NV soil. The depletion/increase was calculated by substracting the Ni concentration in each fraction (in mg kg^{-1}) in the rhizosphere soil from the corresponding concentration in the NV soil. A significant decrease/increase compared to NV soil is denoted by an asterisk and a significant difference between plants is denoted with "≠" ($p \leq 0.05$).

Among the remaining fractions, Ni was mainly associated with Mn oxides (F3; 5 to 6% of total Ni), or in the case of the hyperaccumulator, organic matter-bound (4%). Figure 4.2b shows the change (decrease/increase) in Ni concentrations of each fraction compared to the NV soil. Both plant species significantly increased F1 (mobile fraction) (from 27.8 mg Ni kg^{-1} in NV soils to 55.4 mg Ni kg^{-1} and 56.8 mg Ni kg^{-1} in the rhizosphere of *A. pintodasilvae* and *H. lanatus*, respectively) ($p \leq 0.05$). Again, both plant species significantly increased the readily available Ni fraction (F2) but this was more pronounced in the hyperaccumulator: the F2 Ni fraction increased from 23.0 mg kg^{-1} in NV soil to 82.2 mg kg^{-1} and 56.8 mg kg^{-1} in the rhizosphere of the *A. pintodasilvae* and *H. lanatus*, respectively. Moreover, the hyperaccumulator significantly increased Ni bound to organic matter (F4) (from 69.1 mg kg^{-1} to 157.0 mg kg^{-1}) and Ni bound to amorphous Fe oxides (F5) (from 6.4 mg kg^{-1} to 10.9 mg kg^{-1}), but these plant-induced changes were not observed in the Ni-excluder ($p < 0.05$).

Concentrations of other major elements are presented in Table 4.3 (Ca $(NO_3)_2$-, $Sr(NO_3)_2$- and NH_4NO_3 (F1)-extractable elements) and Table 4.4 (C_{DGT}). In general, enrichment in most elements was observed in the rhizosphere of the hyperaccumulator compared to NV or to *H. lanatus* (except for Mg which was higher in the rhizosphere of *H. lanatus*) (Table 4.3). NH_4NO_3-extractable (F1) Ca concentrations were up to 3-fold higher in the rhizosphere of the hyperaccumulator than in NV soil, and up to 2.5-fold higher than in the rhizosphere of *H. lanatus*. Similarly, available K (extracted with $Ca(NO_3)_2$, $Sr(NO_3)_2$ and NH_4NO_3 (F1)) was significantly higher in the hyperaccumulator: $Ca(NO_3)_2$-extractable concentrations of this nutrient were up to 5-fold higher than in NV soils or the rhizosphere of *H. lanatus*. The highest concentrations of available Co, Mn, Fe (only in the case of NH_4NO_3-extractable concentrations) and Al were also recorded in the rhizosphere of the hyperaccumulator (although this increase was only significant for Mn). The C_{DGT} concentrations of the trace elements, Ni, Mn, Co and Al, were significantly higher in *A. pintodasilvae* rhizosphere soil compared to either NV soil or the rhizosphere of *H. lanatus* (Table 3.4). In contrast, C_{DGT} Fe concentrations were significantly higher in the rhizosphere of *H. lanatus*. This was the case after 24h of deployment, and the same trend was generally maintained until the longest deployment time. After 24h deployment, DGT-labile Ni (C_{DGT}) in the rhizosphere of the hyperaccumulator was up to 5-fold higher than NV soil and 2.5-fold higher than in the rhizosphere of *H. lanatus*. Similarly, in the rhizosphere soil of the hyperaccumulator, DGT-labile Mn, Co and Al concentra-

Table 4.3. Concentration of available Ca, K, Mg, Mn, Fe, Co, Al determined through conventional soil extractions. Different letters indicate significant differences between soils ($p < 0.05$).

		Ca	K	Mg	Mn	Fe	Co	Al
		mg kg^{-1} (mean ± *SE*)						
$Ca(NO_3)_2$	NV	nd	15.2 ± *2.1 b*	665.6 ± *6.4 c*	1.1 ± *0.1 b*	9.7 ± *2.6 a*	0.06 ± *0.01 a*	3.5 ± *2.0 a*
	A. pintodasilvae	nd	70.2 ± *0.9 a*	776.5 ± *18.0 b*	2.0 ± *0.2 a*	5.1 ± *0.5 a*	0.08 ± *0.02 a*	3.0 ± *0.3 a*
	H. lanatus	nd	15.4 ± *0.2 b*	958.5 ± *5.1 a*	0.8 ± *0.1 b*	7.8 ± *2.4 a*	0.04 ± *0.01 a*	1.9 ± *0.4 a*
$Sr(NO_3)_2$	NV	220.9 ± *4.7 c*	18.6 ± *2.0 b*	639.8 ± *14.4 c*	1.1 ± *0.1 b*	7.3 ± *1.0 a*	0.09 ± *0.01 a*	4.4 ± *0.8 a*
	A. pintodasilvae	708.1 ± *11.2 a*	75.7 ± *2.7 a*	715.9 ± *12.2 b*	1.7 ± *0.1 a*	6.8 ± *1.1 a*	0.09 ± *0.01 a*	4.8 ± *0.6 a*
	H. lanatus	393.3 ± *27.6 b*	18.4 ± *1.0 b*	906.3 ± *5.9 a*	0.9 ± *0.2 b*	8.1 ± *0.5 a*	0.10 ± *0.00 a*	3.6 ± *0.8 a*
NH_4NO_3	NV	372.8 ± *118.0 b*	44.4 ± *2.8 c*	1034.7 ± *18.2 c*	5.6 ± *1.4 b*	4.9 ± *1.6 a*	0.40 ± *0.03 a*	4.1 ± *1.3 a*
	A. pintodasilvae	1135.2 ± *205.0 a*	162.6 ± *4.2 a*	1209.2 ± *56.6 b*	14.9 ± *1.5 a*	9.4 ± *3.4 a*	0.68 ± *0.11 a*	6.6 ± *0.7 a*
	H. lanatus	449.7 ± *3.2 b*	74.3 ± *4.9 b*	1582.2 ± *29.8 a*	16.9 ± *0.7 a*	4.9 ± *0.3 a*	0.60 ± *0.16 a*	3.8 ± *0.5 a*

nd: not determined

Table 4.4. Concentrations (µg L^{-1}) of C_{DGT} Ni, Mn, Fe, Co and Al determined at the four deployment times. Different letters indicate significantly differences between soils ($p < 0.05$).

		Ni	Mn	Fe	Co	Al
		C_{DGT} (µL L^{-1})				
24 h	NV	141.6 ± *10.9* c	157.9 ± *43.7* c	22.0 ± *3.8* c	2.3 ± *1.0* c	6.7 ± *1.1* b
	A. pintodasilvae	668.8 ± *17.3* a	3783.0 ± *78.0* a	70.9 ± *12.8* b	65.2 ± *1.6* a	60.6 ± *4.8* a
	H. lanatus	271.0 ± *19.0* b	2339.5 ± *144.3* b	159.5 ± *14.2* a	52.8 ± *4.1* b	42.4 ± *3.5* b
62 h	NV	133.1 ± *4.9* c	51.4 ± *4.3* c	10.0 ± *2.8* b	1.5 ± *0.2* b	2.8 ± *0.7* b
	A. pintodasilvae	409.1 ± *22.2* a	2137.0 ± *98.5* a	12.2 ± *3.8* b	41.0 ± *2.5* a	16.7 ± *2.7* a
	H. lanatus	232.0 ± *11.1* b	1581.6 ± *62.2* b	73.2 ± *12.4* a	42.9 ± *2.6* a	19.1 ± *3.4* a
168 h	NV	94.7 ± *7.5* b	13.6 ± *1.2* c	4.9 ± *1.0* b	0.7 ± *0.1* b	2.0 ± *0.3* a
	A. pintodasilvae	285.9 ± *4.7* a	1163.4 ± *101.6* a	28.6 ± *10.7* b	27.7 ± *2.0* a	8.8 ± *2.6* a
	H. lanatus	227.0 ± *64.9* a	307.8 ± *43.3*b	121.5 ± *3.9* a	27.2 ± *4.0* a	18.5 ± *6.5* a
264 h	NV	82.4 ± *7.1* c	5.0 ± *0.6* b	11.3 ± *3.9* a	0.5 ± *0.1* c	3.4 ± *1.2* a
	A. pintodasilvae	234.9 ± *8.1* a	653.1 ± *113.1* a	8.3 ± *2.2* a	18.3 ± *0.6* a	3.1 ± *0.6* a
	H. lanatus	155.3 ± *6.1* b	55.9 ± *7.2* b	12.4 ± *4.6* a	12.9 ± *1.0* b	3.7 ± *1.1* a

tions were up 24-, 28- and 10-fold higher than NV soil, and 2-, 1.2-, and 1.4-fold higher than in the *H. lanatus* rhizosphere. On the other hand, DGT-labile Fe was 2.2-fold higher in the rhizosphere of *H. lanatus* than of the hyperaccumulator. For Ni, Mn and Co the same effects were observed at longer deployment times (Table 3.4). DGT labile Ni was positively correlated with Mn, Co and Al (R^2=0.6-0.8).

Soil Ni replenishment kinetics

Figure 4.3a shows the accumulated mass of Ni on the resin gel (mol cm^{-2}) with deployment time (at 24, 72, 168 and 264 h). In all soils the accumulated Ni mass increased with increasing deployment time. This increase was more pronounced in rhizosphere soils than NV soil, and in particular, in the rhizosphere of the hyperaccumulator.

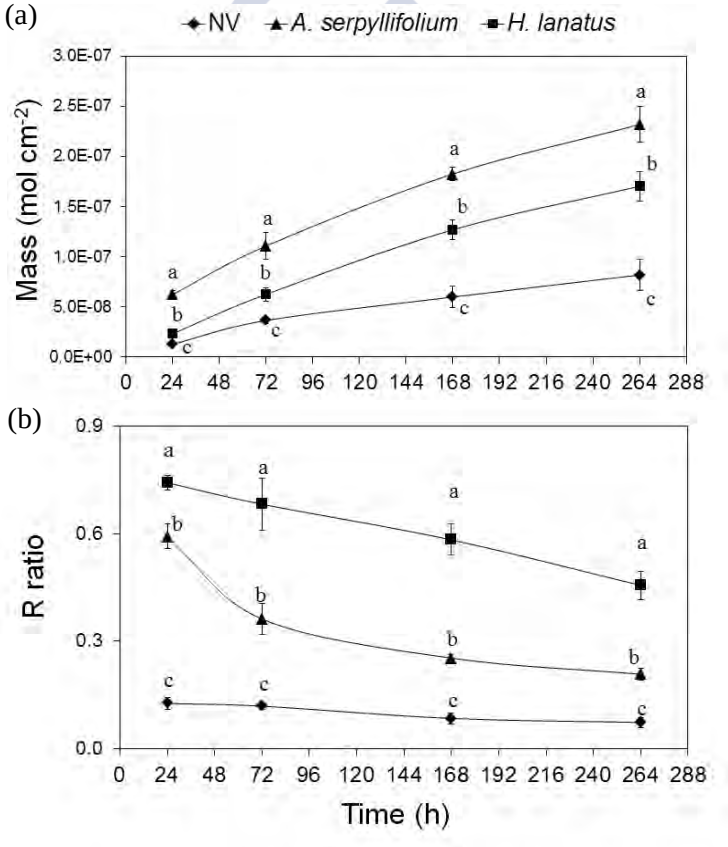


Figure 4.3. Measured accumulated Ni mass in the resin gel (mol cm^{-2}) and the Ni *R* ratio (C_{DGT} / Csol) with deployment time (24, 62, 168, 264 h). Different letters indicate significant differences between soils at each deployment time ($p < 0.05$).

The accumulated Ni mass in *A. pintodasilvae* rhizosphere soil was significantly higher at all deployment times (increasing from from 6.25E-08 mol Ni cm^{-2} after 24h to 2.32E-07 mol Ni cm^{-2} after 264h) than in both *H. lanatus* rhizosphere soil (increasing from 2.34E-08 mol Ni cm^{-2} to 1.70E-0.7 mol Ni cm^{-2}) and the NV soil (increasing from 1.30E-0.8 mol Ni cm^{-2} to 8.19E-08 mol Ni cm^{-2}). Figure 4.3b shows the change in the *R* ratio (C_{DGT}/C_{sol}) values with deployment time. *H. lanatus* rhizosphere soil presented significantly higher *R* ratio values than either *A. pintodasilvae* rhizosphere or NV soil.

The R ratio was 0.74, 0.59 and 0.13 after 24 h in *H. lanatus*, *A. pintodasilvae* or NV soil, respectively. R ratios of the NV soil were always significantly lower than those of either rhizosphere soil ($p < 0.05$). In rhizosphere soils, there was a progressive depletion with increasing deployment time (from 24 h to 264 h), while in NV soil there was almost no change with time (R ratios in NV soil were 0.13 after 24 h and fell to 0.07 after 264 h) (Figure 4. 3b). For *H. lanatus*, the R ratio fell from 0.74 at 24 h to 0.46 at 264 h, while in *A. pintodasilvae* the R ratio fell from 0.59 at 24 h to 0.21 at 264 h (Figure 4.3b).

The kinetics of metal resupply were obtained from the best fitting curve of experimental R values against time using the 2D DIFS software. The soil subdomain in the 2D DIFS model extends 40 mm from the DGT probe interface. Therefore, the accuracy with which the model represents the Ni depletion in the soil water in these experiments may not be optimal, since the soil layer was less than 8-10 mm from the probe interface, owing to the small amount of available rhizosphere soil. However, given that only one soil was used and the amount used was consistent between deployments, the model can be used to make qualitative comparisons between the resupply dynamics of non-vegetated soil and the two plant rhizospheres. Absolute values of K_D and T_C must therefore be considered with caution.

Figure 4.4 shows the 2D DIFS model simulation of R for Ni over time: the continuous line shows the best fitting combination of K_D and *T*c, and the two dotted lines show the adjustment after modifying this combination by a maximum of 30%. While these cannot be used as absolute values of K_D and T_C in these soils, they do allow us to make qualitative comparisons of the Ni resupply dynamics between soils before and after modification by the two plant species. The model results suggest that the solid phase reservoir of labile Ni in the NV soils was very small with a long response time before root growth. Ni resupply to the DGT probe here occurs mainly through diffusion (small labile Ni pool (K_D of 5) and a

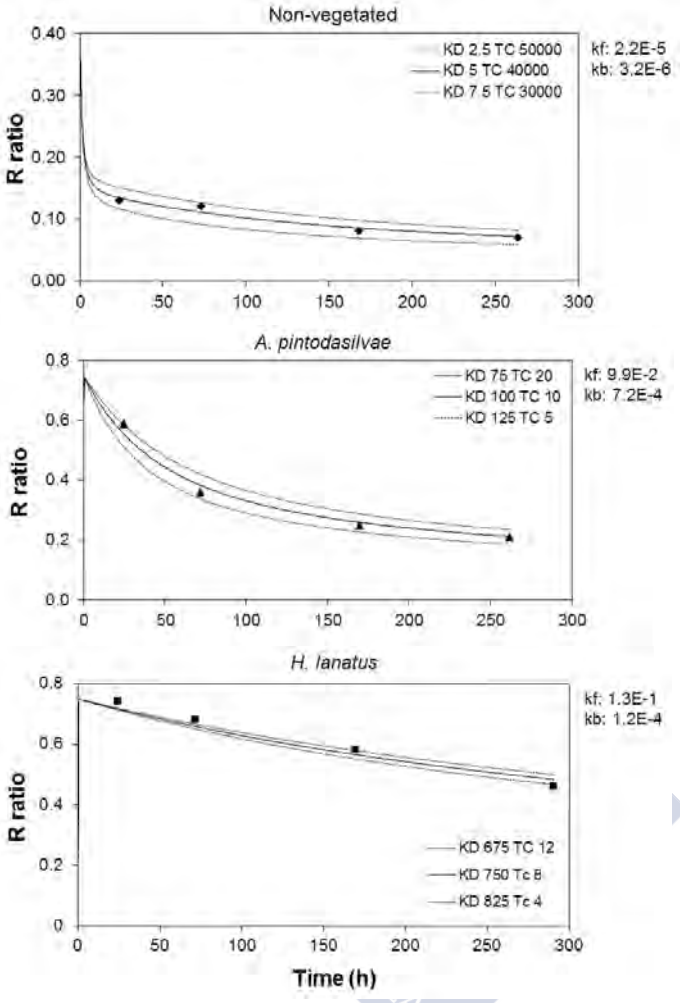


Figure 4.4. Experimental data and 2D DIFS model simulation of *R* for Ni with error margins (T_C and K_D were changed by 30% from the best model fit). Kf (sorption rate; s^{-1}) and Kb (desorption rate; s^{-1})

high response time (T_C of 40000); E = 1.14). The modelled *R* values in the *A. pintodasilvae* rhizosphere soil suggest that the solid phase reservoir of labile Ni has increased, and the resupply from this solid phase is faster than in the NV soil (K_D of 100 and T_C of 10; E = 4.36). Ni resupply to the DGT device in this soil is likely to be partially sustained by a labile pool of Ni sorbed to solid phases. The largest model-estimated reservoir of labile solid phase-bound Ni was observed in the *H. lanatus* rhizosphere, Ni resupply to the DGT device here is almost completely sustained by the solid phase (K_D 750 and T_C 8; E = 4.28).

Chemical Imaging of DGT-available Ni along the root

High-resolution imaging of DGT-measured Ni fluxes around *A. pintodasilvae* and *H. lanatus* roots was carried out to show spatial variability of root activities and potential root-induced Ni solubilisation, metal depletion and/or accumulation in the rhizosphere. Figure 4.5 shows the DGT-measured Ni fluxes (proportional to the DGT-available fraction) around a single root of *A. pintodasilvae* or *H. lanatus*. Depletion Ni zones were found along the root axis of both species although the extent of this depletion was slightly higher along the root of the Ni hyperaccumulator.

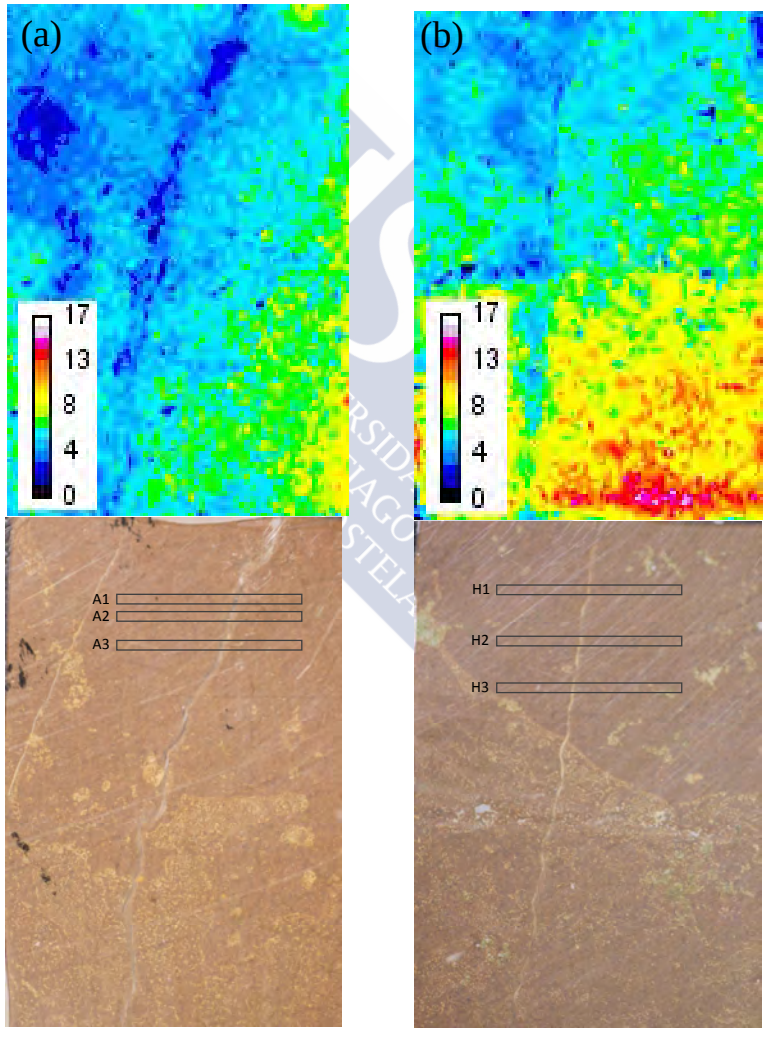


Figure 4.5. High resolution two-dimensional mapping of Ni along the root axis of of *A. pintodasilve* (a) and *H. lanatus* (b). Metals are shown as DGT-measured metal fluxes (pg cm^{-2} s^{-1}). Root cross sections are referred to in the next figure (Figure 4.6.).

Figure 4.6 shows the horizontal profile plot of Ni flux (pg cm^{-2} s^{-1}) over the root cross sections labelled as A1, A2, A3 and A4 (corresponding to *A. pintodasilvae* roots) and profiles labelled as H1, H2 and H3 (corresponding to *H. lanatus* roots). These profiles were chosen as areas of better contact between the soil and DGT gel. DGT-measured Ni fluxes were reduced by up to 3 mm distance from the root surface in the case of *A. pintodasilvae* (line A2) while in *H. lanatus* the depletion area only reached a maximum of 0.8 mm (line H1).

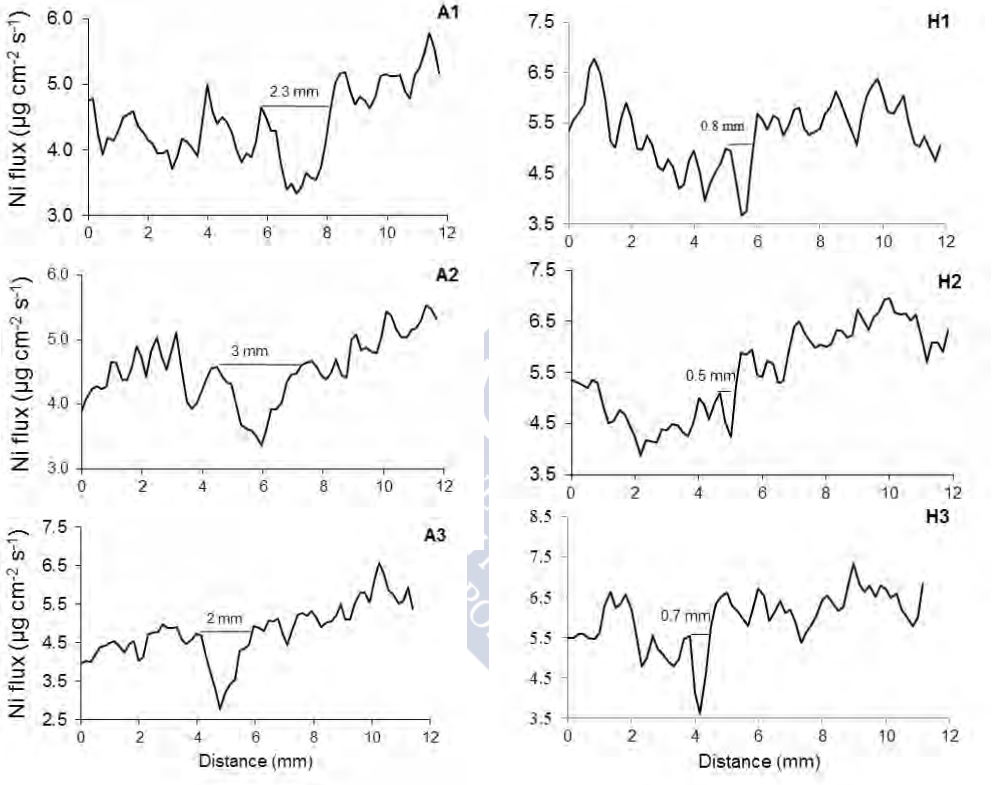


Figure 4.6. Horizontal profile plot of Ni flux (pg cm^{-2} s^{-1}) over root cross sections. Profiles labelled as A1, A2, A3 and A4 correspond to *A. pintodasilvae* roots and profiles labelled as H1, H2 and H3 to *H. lanatus* roots.

4.4. Discussion

Monitoring changes in metal mobility and availability in the rhizosphere of hyperaccumulating plants is a basic requirement for evaluating the efficiency and long-term sustainability of phytomining processes (Fitz and Wenzel, 2002; Wenzel *et al.*, 2004). Moreover, a deeper understanding of the processes operating in the rhizosphere during metal accumulation could help further develop these

metal recovery techniques. In this study, the Ni-hyperaccumulator *A. pintodasilvae* was compared with the non-accumulator *H. lanatus*. Shoot Ni concentrations in *A. pintodasilvae* were similar to values previously reported for this plant species (Álvarez-López *et al.*, 2015; Cabello-Conejo, 2015) and confirmed its ability to hyperaccumulate this metal. In contrast, shoot Ni concentrations in *H. lanatus* were very low (10 mg kg^{-1}), and in this species Ni tended to accumulate in the roots (with a mean root Ni concentration of 397 mg kg^{-1}).

Concentrations of labile Ni (estimated using the different simple extractions and C_{DGT}) were significantly higher in the rhizosphere soils than non-vegetated (NV) soil. Moreover, labile Ni concentrations were higher in the rhizosphere of the hyperaccumulator than the non-accumulator. Soil Ni fractionation was also modified in rhizosphere soils compared to NV soil, and shifts in Ni fractionation towards more plant-available and potentially available fractions were more pronounced in the Ni-hyperaccumulator than the non-accumulator. In the rhizosphere soil of *A. pintodasilvae* a significant increase in the easily mobile Ni fraction, Ni bound to organic matter, and Ni associated with amorphous Fe oxides was observed. The differences in soil Ni availability were more pronounced when using the DGT technique than the simple soil extractions. On the basis of C_{DGT}, Ni availability was 3-fold higher in the rhizosphere of the hyperaccumulator than the non-accumulator, while the corresponding increase in either $Ca(NO_3)_2$- or $Sr(NO_3)_2$-extractable Ni concentrations was only 1.1-fold. Previous studies have also found higher soil Ni availability in the rhizosphere of Ni-hyperaccumulators. Wenzel *et al.* (2003) found soluble (water-extractable) Ni concentrations in the rhizosphere of the Ni-hyperaccumulator *Noccaea goesingensis* collected from the Redlschlag serpentine site (Austria) to be 3- and 2-fold higher than in corresponding NV soil or rhizosphere soil of non-accumulating plant species. Our results support other studies suggesting that the DGT technique can give a more precise estimation of plant-available metal fractions than conventional soil extractions (Zhang *et al.*, 2001; Tandy *et al.*, 2011). Moreover, the DGT technique would appear to be more sensitive to plant-induced changes in soil metal availability and more suitable for differentiating between different plant types e.g. metal (hyper)accumulators vs. excluders.

An increase in metal availability and plant uptake is often associated with a root-induced acidification effect (Hinsinger *et al.*, 2005). However, most studies suggest that root-induced pH changes are not involved in the hyperaccumulation mechanism (Bernal and McGrath, 1994; Knight *et al.*, 1997; McGrath *et al.*,

1997; Luo *et al.*, 2000; McGrath *et al.*, 2001). In agreement, no pH decrease was found in the rhizosphere of *A. pintodasilvae* and even a slight increase was observed. Wenzel *et al.* (2003) also found an increase in soil pH in the rhizosphere of *N. goesingensis* growing in serpentine soils. It is also well known that root exudates can influence metal availability, through other processes than acidification such as chelation, precipitation and redox reactions (Uren and Reisenauer, 1988; Adriano, 2001; Puschenreiter *et al.*, 2003; Tao *et al.*, 2004; Hinsinger *et al.*, 2005). DOC concentrations were higher in the rhizosphere of the hyperaccumulator than the non-accumulator, and labile Ni concentrations were positively correlated with DOC concentration. Previous studies have also related enhanced metal availability at the rhizosphere of metal hyperaccumulators to higher DOC concentrations. The higher concentration of water-extractable Ni in the rhizosphere of *N. goesingensis* compared to non-vegetated soil was correlated with higher DOC concentrations (Wenzel *et al.*, 2003). Using chemical speciation analysis (MINTEQA2), Wenzel *et al.* (2003) suggested that the higher soluble Ni concentration in the hyperaccumulator rhizosphere was driven by the formation of Ni-organic complexes. Moreover, the nature of DOC in the rhizosphere is likely to differ among plant species. Wenzel *et al.* (2003) suggested that DOC in the rhizosphere of hyperaccumulators may form stronger complexes with Ni than DOC derived from bulk soil or the rhizosphere soil of Ni-excluders (such as the *H. lanatus* in this study). Cattani *et al.* (2009) found that DOC released by the As-hyperaccumulator *Pteris vittata* had contrasting effects to that of the As-tolerant *Agrostis capillaris* on As mobility.

Nutrient deficiency is common in serpentine soils and root exudation of organic compounds likely plays a vital role in the mobilisation and uptake of essential elements, such as Ca, P, K or Fe (Marschner, 1995; Neumann and Römheld, 2007; Marschner *et al.*, 2011). The shoot ionome of the two study species clearly showed that the Ni-hyperaccumulator was more effective than the grass species in overcoming the nutrient limiting conditions to which they were both exposed. Shoot macronutrient concentrations (Ca, K, P) and micronutrients (Fe) were significantly higher in *A. pintodasilvae* (between 1.5- and 36-fold higher than in *H. lanatus*). In contrast to *H. lanatus*, the hyperaccumulator was able to notably increase exchangeable Ca and K in the rhizosphere soil (by up to 3- and 5-fold compared to NV soil) and significantly improved the strong nutritional disequilibrium (e.g. the Ca/Mg ratio in the exchange complex increased from 0.15 to up to 0.40). In parallel, C_{DGT} (at 24 h) showed a clear enrichment in elements such

as Al (up to 9-fold), Co (28-fold) Fe (5.5-fold), Mn (24-fold) and Ni (5-fold) in the rhizosphere of this plant compared to NV soils. Most of these increments were maintained with deployment time. An enhanced weathering of primary minerals (such as orthopyroxene or amphibole) by the Ni-hyperaccumulator would easily explain the observed increments in labile concentrations of all these elements. At the same time, mineralisation of soil organic matter present in the rhizosphere of the hyperaccumulator could also be contributing to this nutrient cycling. In consequence, root activity and ligand-induced dissolution of soil minerals would not only lead to the release of essential plant nutrients but also to the Ni associated with these solid phases. The significant increase in the easily mobile Ni fraction, Ni bound to organic matter and to amorphous Fe oxides further supports the more intense weathering activity by the hyperaccumulator and root-induced co-dissolution of Ni-bearing minerals. Antić-Mladenović *et al.* (2011) suggested that the dissolution and precipitation of Fe/Mn oxides, organic matter transformations, and adsorption on solids were important processes controlling Ni solubility in ultramafic soils. Similarly, a study carried out in the ultramafic region of Morais (Iberian Peninsula) revealed that the Ni bioavailability in these soils is linked to the Mn oxide fraction (Alves *et al.*, 2011). Wenzel *et al.* (2003) considered the increase in pH in the rhizosphere of *N. goesingensis* to be consistent with the release of hydroxyl ions during mineral dissolution of Mg/Ni-bearing orthosilicates. These authors also found elevated concentrations of Ca, Mg and K in water extracts of the rhizosphere soil of *N. goesingensis* compared to non-vegetated soil. Fitz *et al.* (2003) also found that increased concentrations of DOC were correlated with enhanced Fe solubility in the rhizosphere of the As-hyperaccumulator *Pteris vittata*. The authors related a DOC-triggered dissolution of iron hydroxide surfaces with an enhanced co-dissolution of As and Fe.

The capacity to pre-concentrate analytes *in situ* and to determine element fluxes instead of equilibrium concentrations like in conventional extraction methods makes DGT highly suitable for studying high resolution element distribution around single roots (Kreuzeder *et al.*, 2015). Ni distribution patterns around roots of the plants cultivated in rhizotrons showed that both plants species depleted soluble Ni probably due to plant uptake, but the extent of this depletion was higher in the rhizosphere of *A. pintodasilvae*. These results appear contradictory to the results of the bulk DGT measurements on field rhizosphere samples. In the latter, rhizosphere soil of adult plants (older than 2 yr) was assessed, whereas in the rhizotron single roots that were only a few days old were investigated. It

can be assumed that the effect of root activity on mineral weathering and Ni availability is more pronounced in the field-collected rhizosphere soils. Our results suggest that at the root tip of very young roots exploring non-vegetated soil, Ni is effectively and quickly depleted, whereas over time (i.e. several months to years) ongoing root activities of the whole root system lead to an overall net mobilisation of Ni in the rhizosphere as a result of the plant's attempts to improve its nutritional status. *A. pintodasilvae* induced a slightly more pronounced depletion zone due to its hyperaccumulation trait, also indicating that the root activity of these plants can influence a larger soil volume than other plant species such as the *H. lanatus* studied here. In spite of its metal excluding nature, *H. lanatus* also induced a Ni depletion zone around the investigated root. This was likely due to the fact that excluder plants also accumulate/adsorb substantial Ni concentrations in their roots and on the root surface. Understanding the exchange of trace metals between the solid and the solution phase is critical when trying to estimate their mobility and bioavailability in the soil (McLaughlin *et al.*, 1998; Harper *et al.*, 2000). The DIFS model can provide information related to the kinetics of metal replenishment by quantifying the relationship between R (C_{DGT}/C_{Sol}), K_D (distribution coefficient between the solid and solution phases) and T_C (response time of the soil to reach equilibrium) (Harper *et al.*, 1998). The accumulated Ni mass in DGT followed the order *A. pintodasilvae*>*H. lanatus*>NV soil, and this was consistent across the full deployment time. The higher accumulated Ni mass from the rhizosphere soil of *A. pintodasilvae* compared to *H. lanatus* is in agreement with its higher labile Ni concentration. Experimental R values at 24 h differed somewhat between the two rhizosphere soils (R= 0.59 (*A. pintodasilvae*) vs. 0.74 (*H. lanatus*), however, the best DIFS model fit for R and deployment time predicted R values to peak at 0.74 and 0.75 for both rhizosphere soils before the 24 h deployment time. This indicates that the resin took up a similar proportion of soluble Ni from both soils in a short time. After this peak, R values progressively decreased with time in both rhizosphere soils, indicating an initially greater Ni flux to the diffusion layer from the soluble phase than from the solid phase. However, the decline in R values was more pronounced in the rhizosphere of the hyperaccumulator than the excluder. The rhizosphere soil of *A. pintodasilvae* effectively buffered Ni concentration in the soil solution at the first deployment time (24 h), but with time the Ni resupply was not sufficient to maintain the high initial concentrations of soluble Ni (1130 $\mu g\ L^{-1}$) in the soil solution (non-steady state case). While in *H. lanatus* rhizosphere soil the lower initial soluble Ni concentration (340 $\mu g\ L^{-1}$) appeared to be more

easily compensated for through replenishment from the solid phase (sustained case). In contrast, in NV soils there was practically no resupply from the solid phase and the data were best-fitted to a diffusive case, which is defined by no exchange reaction of metal between the sorbed phase and the soil solution and characterised by a very low K_D and high *T*c. The predicted K_D values for both plant species were significantly higher than those of the NV soil (K_D = 5), confirming their greater solid-phase labile Ni pool. However, K_D values were higher for the excluder than the hyperaccumulator which indicates that the hyperaccumulator maintains a higher proportion of Ni in the dissolved phase than in the sorbed phase at equilibrium. Whereas the soil response time (*T*c) was similar for both plant species.

The DIFS model also has some limitations, such as the assumption that metal in solution is in a fully labile and mobile form (and not present as metal-complexes that have slow release kinetics and/or are large enough to diffuse slowly) (Lehto *et al.*, 2008). For example, the DIFS model does not directly consider metal complexation with dissolved organic ligands (such as DOC) and some recent studies have found that DGT measurements might be affected when a significant amount of metal is bound by humic substances in solution. This bound metal can have diffusion coefficients as low as 20% of the free metal diffusion coefficient (Lehto *et al.*, 2006b) and the kinetics of metal release from the complex may be slowed down. These organic complexes often diffuse much slower than the metal and therefore can buffer the metal ion concentration, and can be perceived as components of K_D by the DIFS model. As mentioned above, the DOC concentration in the rhizosphere soil of both plant species differed and, moreover, the nature of this DOC is likely to differ between plant species. Plant-specific complexing agents may be present in the rhizosphere soils and are likely to influence the DGT measurements and DIFS predictions: grasses are well known for releasing phytosiderophores for Fe solubilisation (and indeed DGT-labile Fe concentrations were higher in the rhizosphere of *H. lanatus* than the hyperaccumulator). In addition to increasing Fe uptake these chelants can also mobilise other trace metals, in particular Zn, Cu and Ni (Zhang *et al.*, 1991; Awad and Römheld, 2000). Finally, it is worth pointing out that the hyperaccumulator plant clearly modified soil Ni availability and fractionation to a greater extent than the excluder. Although the DIFS model showed that the initial high soluble Ni concentration in the soil solution of this plant could not be sustained by the soil buffering, only reinforces the importance of the hyperaccumulator root activity

and its influence on soil Ni availability. Since the plant was not present this effect is lost, but it can be assumed that in a dynamic system (such as the rhizosphere) the labile Ni concentrations will be sustained over time. Future studies will assess the soil Ni replenishment and kinetics in rhizosphere soils sampled at different time intervals.

4.5. Conclusions

Both plant species influenced soil physicochemical characteristics such as pH, DOC, CEC, Ca/Mg ratio or Ni availability and fractionation. However, plant-induced changes in Ni availability and fractionation were more pronounced in the case of the hyperaccumulator. The DGT technique was a more sensitive tool for highlighting these plant-induced changes than the conventional extractions. Our results suggest that the enhanced Ni availability in the rhizosphere of *A. pintodasilvae* is not due to active metal mobilisation by the hyperaccumulator but rather a consequential effect in response to the plant's efforts to improve nutrition through mineral weathering and/or mineralisation of organic matter. These plants have clearly developed effective mechanisms for improving their nutritive status in these soils. Moreover, it seems clear that DOC plays a fundamental role in determining soil metal availability and further studies are needed to characterise the composition of the root exudates released by both plant species.

DIFS modelling showed a progressive decline in *R* values with time in both rhizosphere soils, however, this was more pronounced in the rhizosphere of the hyperaccumulator than the excluder. The rhizosphere soil of *A. pintodasilvae* effectively buffered Ni concentration in the soil solution at the first deployment time (24 h), but with time Ni resupply was not sufficient to maintain the high initial concentrations of soluble Ni in the soil solution. While in *H. lanatus* rhizosphere soil the lower initial soluble Ni concentration were more easily compensated for through replenishment from the solid phase.

4.6. References

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Chapter 5

Organic amendments for improving biomass production and metal yield of Ni-hyperaccumulating plants.

ABSTRACT

Ni phytomining is a promising technology for Ni recovery from low-grade ores such as ultramafic soils. Metal-hyperaccumulators are good candidates for phytomining due to their extraordinary capacity for Ni accumulation. However, many of these plants produce a low biomass, which makes the use of agronomic techniques for improving their growth necessary. In this study, the Ni hyperaccumulators *Alyssum serpyllifolium* subsp. *lusitanicum*, *A. serpyllifolium* subsp. *malacitanum*, *A. bertolonii* and *Noccaea goesingensis* were evaluated for their Ni phytoextraction efficiency from a Ni-rich serpentine soil. Effects of soil inorganic fertilisation (100:100:125 kg NPK ha^{-1}) and soil organic amendment addition (2.5, 5 or 10% compost) on plant growth and Ni accumulation were determined. All soil treatments greatly improved plant growth, but the highest biomass production was generally found after addition of 2.5 or 5% compost (w/w). The most pronounced beneficial effects were observed for *N. goesingensis*. Total Ni phytoextracted from soils was significantly improved using both soil treatments (inorganic and organic), despite the decrease observed in soil Ni availability and shoot Ni concentrations in compost-amended soils. The most promising results were found using intermediate amount of compost, indicating that these types of organic wastes can be incorporated into phytomining systems.

This study formed part of the following publication:

Álvarez-López V., Prieto-Fernández Á., Cabello-Conejo M.I. and Kidd P.S. (2016). Organic amendments for improving biomass production and metal yield of Ni-hyperaccumulating plants. *Sci Tot Envir* 548-549:370-379.

5.1. Introduction

Metal hyperaccumulator plants present an extreme ability to accumulate trace elements in their harvestable tissues. To date, approximately 500 taxa of metal hyperaccumulators have been identified, of which over 90% accumulate nickel (Ni) (Pollard *et al.*, 2014). The Brassicaceae family, and specifically the genus *Alyssum*, present the greatest number of Ni hyperaccumulators (Pollard *et al.*, 2014). The vast majority of Ni-hyperaccumulating species are endemic to soils developed over ultramafic rocks (naturally rich in metals such as Co, Cr and Ni), and can present up to 1-3% dry weight Ni content in their shoots (Reeves, 2006). This characteristic makes these plants particularly useful for application in phytoextraction or phytomining (Chaney *et al.*, 2010). Phytoextraction techniques are focused on the remediation of degraded or contaminated soils using plants which are able to accumulate high amount of metals in their harvestable plants, while phytomining is the phytoextraction and recovery of metals from the harvested plant biomass for commercial gain (Chaney *et al.*, 2007; Tang *et al.*, 2012). Ni phytomining was proven to be economically feasible in the USA and Canada (Li *et al.*, 2003a; Chaney *et al.*, 2007), and more recently, in Europe (Bani *et al.*, 2007; Bani *et al.*, 2015). To date the Ni-hyperaccumulating *Alyssum* species, *A. murale* and *A. corsicum* (native to Mediterranean serpentine soils), have shown the highest potential application in phytomining processes (Li *et al.*, 2003b; Chaney *et al.*, 2007). Selected plants for Ni phytomining purposes should be able to hyperaccumulate this metal and also to tolerate and thrive under the adverse growth conditions which are frequently present in metal-rich environments (Harris *et al.*, 2009). The use of native plant species has several advantages, both for practical reasons and also as a means of supporting the conservation of serpentine biodiversity. For example, *A. murale* occurs widely as a native weed of agricultural land on ultramafic Vertisols in Albania, and was successfully implemented in phytomining cropping systems in the Albanian regions of Pojskë and Prrenjas (Bani *et al.*, 2007; Bani *et al.*, 2015). However, a low biomass production can be an important bottleneck limiting the practical application of hyperaccumulators in phytomining (Robinson *et al.*, 1997; McGrath and Zhao, 2003; Mench *et al.*, 2010; Kidd *et al.*, 2015).

Over the last decade several authors have highlighted the need to optimise the phytomining process using appropriate agronomic and crop management practices, such as pH adjustments, fertilisation regimes, design of cropping

patterns (co-cropping or rotation), bioaugmentation using plant-associated microbial inoculants, or the use of plant growth regulators (PGRs) (Chaney *et al.*, 2007; Tang *et al.*, 2012; Cabello-Conejo *et al.*, 2014a; Cabello-Conejo *et al.*, 2014b; Bani *et al.*, 2015; Kidd *et al.*, 2015; Durand *et al.*, 2016). In fact, the concept of agromining was introduced by van der Ent *et al.* (2015) to emphasise that phytomining forms part of an integrated agricultural chain. Several studies have shown the importance of soil fertilisation to increase plant yields and phytoextracted Ni. In field-scale experiments in serpentine soils, Bani *et al.* (2015) found a high positive response in biomass production of *A. murale* using NPK and Ca fertilisers. Robinson *et al.* (1997) enhanced the biomass of *A. bertolonii* with NPK fertilisers by 3-fold. Inorganic fertilisation also increased the biomass of *A. serpyllifolium* ssp. *lusitanicum* growing on a mine-soil by a factor of 10; as a consequence the total Ni phytoextracted experimented a 6-fold increase (Kidd and Monterroso, 2005).

Municipal wastes such as sewage sludges (biosolids) have been used as an amendment to agricultural soils for many years now, and are an economically attractive alternative to disposal through landfill and incineration. However, few studies have considered the use of organic soil amendments to increase Ni phytomining efficiency. Ultramafic soils are characterised by a low fertility, low water holding capacity and poor structure. Compost addition could be a useful means of increasing soil fertility and soil structure (Bernal *et al.*, 2007; Fagnano *et al.*, 2011; Martínez-Fernández *et al.*, 2014). Moreover, the use of composted solid organic residues, such as municipal solid wastes (MSW) or sewage sludge, also constitutes a means of recycling waste products, thus contributing towards the objectives of the EU's Zero Waste Policy (2013).

The objective of this study was to was to evaluate the use of composted MSW as a means of increasing the phytoextraction efficiency, of different Ni hyperaccumulating plants grown in ultramafic soils. The effects of different fertilisation regimes (compost addition or mineral fertilisers) on biomass production and Ni removal of the hyperaccumulators, *Alyssum serpyllifolium* subsp. *lusitanicum* (two populations), *A. serpyllifolium* subsp. *malacitanum* (two populations), *A. bertolonii* (one population) and *Noccaea goesingensis* (one population), were compared.

5.2. Materials and methods

Plant species, study soil and experimental set-up

Soil was collected from the ultramafic area of Barazón (Galicia) in NW Spain (where *A. serpyllifolium* subsp. *lusitanicum* is a native species). Soils were air-dried, sieved through an 8-mm stainless steel sieve and homogenised. Soil treatments included untreated control (no amendment), inorganic fertilisation (NPK), and different rates of an organic amendment addition (commercial compost based on municipal solid wastes (MSW)). Inorganic fertilisers were added as pure chemical reagents: N was added at 100 kg ha^{-1} as NH_4NO_3, and P and K were added as KH_2PO_4 at 100 and 125 kg ha^{-1}, respectively (based on a soil depth of 20 cm and bulk density of 1.1 g cm^{-3}). The compost amendment was applied at three different rates of addition: 2.5%, 5% and 10% w/w. The commercial compost used in this study was provided by a local SME company dedicated to the recycling of waste products (Tratamientos Ecológicos del Noroeste (TEN) s.l.) and is primarily based on municipal sewage sludges and wood chippings. The main properties of the compost are a slightly acidic pH (6.2), high amount of nutrients such as available P (P_{olsen}: 425 mg kg^{-1}), organic C (22.2%) or total N (1.6%), and high cation exchange capacity (CEC: 57.6 $cmol_c$ kg^{-1}). The CEC was dominated by Ca (40.4 $cmol_c$ kg^{-1}), and exchangeable K was 4.4 $cmol_c$ kg^{-1}. After incorporation of amendments the soils were adjusted to 70% of their water holding capacity (which was measured according the method of Guitián and Carballas (1976)) and left for 2 weeks to attain equilibrium, after which time aliquots were taken of each treatment for soil analyses.

Seeds of *Alyssum serpyllifolium* subsp. *lusitanicum* Dudley and P. Silva (hereafter referred to as *A. pintodasilvae*) from Barazón (NW Spain) and Samil (Trás-Os-Montes, NE Portugal), *A. serpyllifolium* subsp. *malacitanum* Rivas Goday (hereafter referred to as *A. malacitanum*) from Sierra Bermeja and Sierra de Aguas (S Spain), *Alyssum bertolonii* subsp. *bertolonii* Desv from Imprunetta (Italy), and *Noccaea goesingensis* Hálácsy from Redlschlag (E Austria) were collected and germinated on a 2:1 perlite:quartz mixture (2:1 v/v) in a growth chamber under controlled conditions (temperature 22-25ºC, PPFD (Photosynthetic photon flux density) of 190 μmol m^{-2} s^{-1}, under a 16/8 h light/dark cycle). Seeds were watered daily with deionised water until germination and thereafter with a Ni -rich serpentine-like macro-nutrient solution which consisted of 2 mM $MgSO_4$, 0.8 mM $Ca(NO_3)_2$, 2.5 mM KNO_3, 0.1 mM K_2HPO_4, 20μM FeEDDHA, 10 μM

H_3BO_3, 2 μM $MnCl_2$, 1 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.2 μM Na_2MoO_4 and 300 μM $NiSO_4$ (based on Chaney *et al.* (2008)). Plastic pots were filled with 1 kg of soil and one four-week-old seedling of each of the six tested taxa was transplanted to each pot. Five replicate pots were established for each soil treatment (untreated, NPK, 2.5%, 5% and 10%) and plants were grown in an environmentally controlled growth chamber for five months (temperature 22-25ºC, PPFD of 190 μmol m^{-2} s^{-1}, under a 16/8 h light/dark cycle).

Plant and soil analysis

General physicochemical properties and the total and available metal concentrations of all soils were determined before planting. Soil pH was measured in H_2O using a 1:2.5 soil:solution ratio. Total C and N were analysed by combustion with a CHN analyser (Model CHN-1000, LECO Corp., St Joseph, MI). Available P was determined by Olsen's $NaHCO_3$ method (Olsen *et al.*, 1954). Exchangeable cations were extracted with 1M NH_4Cl (1:20 soil:solution ratio) after 16 hours of equilibration, and Co and Ni availability was evaluated by extraction with 10 mM $Sr(NO_3)_2$ in a 1:4 soil:solution ratio after 2h shaking (Everhart *et al.*, 2006). Soils (0.25 g) were digested in a 2:1 mixture of concentrated HNO_3:HCl (65% and 37% respectively and trace analysis grade). Blanks and soil reference materials (ultramafic rock SO-3, Canadian Certified Reference Materials Project) were also included in each digestion batch to ensure quality control. After plant growth, soil Ni availability was evaluated by extraction with $Sr(NO_3)_2$. All these soil extracts and the corresponding blanks were then filtered through cellulose paper (Filter-Laboratory 1242, 90-mm diameter, 7-9 μm pore) and analysed by simultaneous inductively coupled plasma optical emission spectrometry (ICP-OES, Varian Vista Pro, Australia).

At harvest, plants were thoroughly washed with pressurised tap water to remove any adhering soil particles, and rinsed in deionised water. Shoots and roots were separated, dried for 48 h at 40 °C and weighed to determine dry weight (DW) biomass. Plant tissues (0.5 g) were digested in a 2:1 HNO_3:HCl mixture and Ca, Fe, K, Mg, Ni, and P were measured also by ICP-OES. Data were expressed in mg kg^{-1} DW plant material. Blanks and reference materials for vegetation (hay powder No. 129, Community Bureau of References, EU) were also included in each digestion batch.

The ability of the plants to bioconcentrate Ni in their aboveground biomass (Bioconcentration Factor, BCF) was calculated as the ratio of the shoot Ni

concentration and the $Sr(NO_3)_2$-extractable concentration in the soil. Total Ni phytoextracted (Ni yield) was calculated as the product of the shoot DW yield and the shoot Ni concentration.

Statistical analyses

Significant effects of soil treatments on soil properties, plant biomass production, ionome, metal accumulation, and metal removal were determined by one-way ANOVA followed by the "post-hoc" Tukey test. All statistical analyses were performed with *SPSS 21.0* software (SPSS Inc., Chicago, IL).

5.3. Results

Effect of fertilisation and compost amendments on soil physicochemical properties

Table 5.1 shows the physicochemical properties of all the soil treatments before planting. Untreated soils presented a slightly acidic pH (5.8), low amount of available P (7.3 mg kg^{-1}) and low total C and N content (3.03% and 0.16% respectively). The cation exchange capacity was low (6.6 $cmol_c$ kg^{-1}) and the Ca/Mg ratio lower than 1. Total metal content was high for Co (73 mg kg^{-1}), Ni (927 mg kg^{-1}) and Cr (1297 mg kg^{-1}) and metal availability. Exchangeable Ni and Co represented approximately 1-2% of the total concentration and $Sr(NO_3)_2$-extractable Ni and Co <1% (Table 5.1).

The main change induced by the addition of inorganic fertilisers was a significant increase in available P (from 7.3 mg kg^{-1} to 22.1 mg kg^{-1}; $p < 0.05$). In addition, a decrease in the total Ni concentration was observed (from 927 mg kg^{-1} to 819 mg kg^{-1}; $p < 0.05$). Compost addition (at all three rates) significantly increased total C and N content, available P, cation exchange capacity (CEC) only significant for 10% ($p < 0.05$), and also improved the Ca/Mg ratio (reaching close to 1 in the 10% compost treatment) ($p < 0.05$). These effects of compost on soil properties were generally more pronounced with an increase in the rate of addition; for example, available P and CEC increased from 18.1 mg kg^{-1} and 2.7 $cmol_c$ kg^{-1} in 2.5% treatment to 97.5 mg kg^{-1} and 11.9 $cmol_c$ kg^{-1} in the 10% treatment, respectively. Exchangeable K ($cmol_c$ kg^{-1}) also increased from 0.13 in the untreated soil to 0.47 after inorganic fertilisation and up to 1.54 after 10% of compost addition. In contrast, amending with compost tended to decrease the total soil metal concentrations, and this decrease was significant in the 2.5% and 10%

Table 5.1. Physicochemical properties of the untreated and treated serpentine soil before planting of *Alyssum* (sub)species and *Noccaea goesingensis*. Different letters indicate significant differences ($p < 0.05$) between treatments.

		Soil treatments			
			Compost		
	Untreated	NPK	2.5%	5%	10%
pH_{H2O}	5.8 ± *0.01* b	5.7 ± *0.02* c	6.2 ± *0.03* a	5.5 ± *0.01* d	5.7 ± *0.01* c
pH_{KCl}	4.9 ± *0.04* bc	4.8 ± *0.01* c	5.1 ± *0.15* bc	5.2 ± *0.02* ab	5.5 ± *0.04* a
P olsen (mg kg^{-1})	7.3 ± *0.86* d	22.1 ± *1.24* c	18.1 ± *0.75* c	28.8 ± *0.44* b	97.5 ± *2.39* a
CEC ($cmol_c$ kg^{-1})	6.6 ± *1.95* b	8.1 ± *0.19* b	8.6 ± *0.65* b	10.9 ± *2.88* b	23.8 ± *1.94* a
Exchangeable K ($cmol_c$ kg^{-1})	0.13 ± *0.03* c	0.47 ± *0.02* bc	0.54 ± *0.03* bc	0.61 ± *0.14* b	1.54 ± *0.15* a
Exchangeable Ca ($cmol_c$ kg^{-1})	0.50 ± *0.12* c	0.54 ± *0.03* c	2.69 ± *0.17* bc	4.47 ± *1.24* b	11.90 ± *1.01* a
Ca / Mg	0.09 ± *0.01* b	0.08 ± *0.00* b	0.51 ± *0.02* a	0.78 ± *0.04* a	1.20 ± *0.04* a
% C	3.03 ± *0.01* d	2.74 ± *0.02* e	3.68 ± *0.02* c	4.11 ± *0.06* b	6.17 ± *0.05* a
% N	0.16 ± *0.01* c	0.14 ± *0.01* c	0.23 ± *0.00* b	0.24 ± *0.01* b	0.41 ± *0.01* a
Total metal concentration (mg kg^{-1})					
Co	73.1 ± *0.5* a	69.6 ± *1.4* ab	62.2 ± *1.3* b	71.8 ± *1.4* a	62.0 ± *3.8* b
Ni	937.0 ± *7.3* a	818.8 ± *14.0* bc	753.5 ± *4.5* cd	878.6 ± *2.4* ab	707.3 ± *42.7* d
Cr	1297.6 ± *18.9* a	1200.9 ± *26.8* a	1203.1 ± *17.6* a	1218.7 ± *12.9* a	965.2 ± *43.7* b
NH_4Cl-extractable metal concentration (mg kg^{-1})					
Co	0.75 ± *0.20* ab	0.91 ± *0.07* a	0.63 ± *0.04* ab	0.56 ± *0.07*ab	0.29 ± *0.03* b
Ni	20.8 ± *4.2* a	21.9 ± *0.5* a	6.7 ± *0.6* b	6.8 ± *0.8* b	1.9 ± *0.1* b
$Sr(NO_3)_2$-extractable metal concentration (mg kg^{-1})					
Co	0.22 ± *0.01* a	0.25 ± *0.01* a	0.09 ± *0.01* b	0.14 ± *0.01* b	0.03 ± *0.01* c
Ni	3.43 ± *0.04* a	3.23 ± *0.04* b	0.95 ± *0.01* d	1.22 ± *0.04* c	0.57 ± *0.02* e

compost treatments (Table 5.1). In the case of Ni, the total concentration was reduced from 937 mg kg^{-1} in untreated soil to 707.3 mg kg^{-1} in the 10% compost treatment. There was also a strong reduction in Ni availability: NH_4Cl-extractable Ni concentrations decreased by 3-, 3- and 11-fold after addition of 2.5%, 5% and 10% compost, respectively, while in the same treatments $Sr(NO_3)_2$-extractable Ni concentrations decreased by 4-, 3- and 6-fold. A similar decrease was observed in Co (1.2 to 2.6-fold for NH_4Cl-extractable concentrations and 2 to 7-fold for $Sr(NO_3)_2$-extractable concentrations).

Plant biomass production, shoot Ni concentration, Ni yield and bioconcentration factor (BCF) in untreated soils

Table 5.2 shows the shoot DW, Ni concentrations, Ni yield and Ni BCF of the six hyperaccumulator plants growing in the untreated soil. No significant differences in biomass production were found between species. Plant growth was poor and shoot DW ranged from 11.6 mg $plant^{-1}$ in *A. pintodasilvae* (Samil population) to 33.8 mg $plant^{-1}$ in *N. goesingensis*, following the decreasing order: *N. goesingensis* > *A. bertolonii* > *A. malacitanum* (S. Bermeja) = *A. pintodasilvae* (Barazón) > *A malacitanum* (S. Aguas) > *A. pintodasilvae* (Samil). Regarding shoot Ni concentrations, the S. Bermeja population of *A. malacitanum* presented the highest concentrations (with a mean concentration of 9523 mg Ni kg^{-1}) and the Barazón population of *A. pintodasilvae* the lowest (5714 mg Ni kg^{-1}). Some differences were observed (although not always significant) in Ni removal (Ni yield) between populations and/or species: *A.bertolonii* and *A. malacitanum* (S. Bermeja)

Table 5.2. Mean shoot biomass, shoot Ni concentration, Ni BCF and Ni yield of the different Ni-hyperaccumulating plants in the untreated soil. Different letters indicate significant differences ($p < 0.05$; $n = 5$) between plant (sub)species.

		Shoot DW (mg $plant^{-1}$)	Ni (mg kg^{-1})	Ni yield (mg $plant^{-1}$)	BCF x 10^2
		Mean ± *SE*	Mean ± *SE*	Mean ± *SE*	Mean ± *SE*
A.pintodasilvae	Barazón	29.2 ± *11.2* a	5713± *647* b	148 ± *39* a	16.4 ± *1.6* c
	Samil	11.6 ± 3.1 a	6665± *1304* ab	117 ± *48* a	19.2 ± *3.6* bc
A. malacitanum	S. Bermeja	29.6 ± 3.5 a	9527± *815* a	279 ± *33*a	27.6 ± *1.7* a
	S. Aguas	27.8 ± 3.5 a	5178± *682* b	152 ± *34* a	15.3 ± *13.2* c
A. bertolonii		33.3 ± 5.2 a	8727± *688* ab	299 ± *61* a	25.6 ± *1.5* ab
Noccaea goesingensis		33.8 ± 4.0 a	6241± *759* ab	211 ± *36* a	18.9 ± *1.2* bc

showed the highest Ni yields (mean values of 299 and 279 mg plant^{-1}, respectively), while the other plants extracted between 117 mg plant^{-1} (*A. pintodasilvae* from Samil) to 152 mg plant^{-1} (*A. malacitanum* from S. Aguas).

All the plant species presented extremely high BCF (based on the $Sr(NO_3)_2$-extractable Ni fraction) and values ranged from 15.3 x 10^2 in *A. malacitanum* (S. Aguas) to up to 27.5 x 10^2 in *A. malacitanum* (S. Bermeja).

Effect of fertilisation and compost amendments on plant biomass production

All the soil treatments increased (although not always significantly) plant growth and biomass production, and this effect was most extreme in *N. goesingensis* where both inorganic fertilisation and compost addition had pronounced beneficial effects (Figure 5.1). In general, and for all three *Alyssum* species, the lowest improvement in shoot biomass production was observed after NPK fertilisation. In this case, the most pronounced effect was observed in the S. Bermeja population of *A. malacitanum* (DW yield increased by 9-fold; from 0.03 g plant^{-1} to 0.28 g plant^{-1}; $p > 0.05$) and in both populations of *A. pintodasilvae* (DW yield increased from 0.01 g plant^{-1} to 0.24 g plant^{-1} (24-fold) and from 0.03 g plant^{-1} to 0.30 g plant^{-1} (10-fold) in Samil and Barazón respectively; $p > 0.05$). In contrast, in the case of *N. goesingensis* the NPK treatment increased shoot DW from 0.03 g plant^{-1} to 2.86 g plant^{-1} (representing an increase of up to 95-fold; $p < 0.05$).

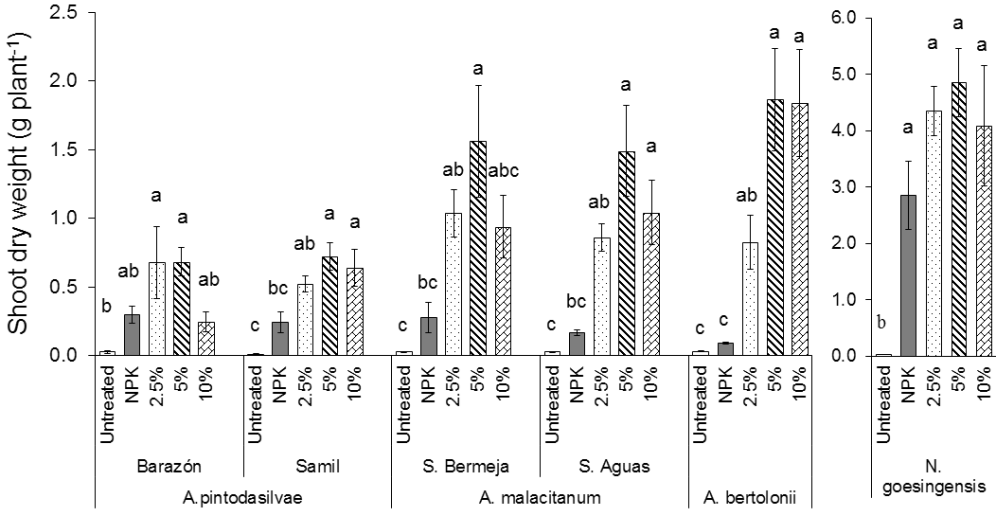


Figure 5.1. Effects of soil treatments (inorganic fertilisation (NPK) and organic amendment (addition rates of 2.5, 5 and 10%) on biomass production of the different Ni-hyperaccumulators. Different letters above bars indicate significant differences between treatments for each plant ($p < 0.05$; $n = 5$). Note that graphs use a different y-axis scale.

After compost addition, *N. goesingensis* presented again the highest response for all rates of addition, and DW yield was increased by up to 162-fold compared to yields obtained in untreated soil after addition of 5% compost ($p < 0.05$). In general, all rates of addition had a beneficial effect on biomass production, but the most pronounced increase in DW yields was obtained with 2.5% or 5% compost. The mean biomass of all plants grown in untreated soil was 0.03 g plant^{-1}, and this increased in the 5% compost treatment to up to 0.68 g plant^{-1} and 0.73 g plant^{-1} in *A. pintodasilvae* (Barazón and Samil populations, respectively; $p < 0.05$), 1.56 g plant^{-1} and 1.49 g plant^{-1} in *A. malacitanum* (S. Bermeja and S. Aguas populations, respectively; $p < 0.05$), 1.87 g plant^{-1} in *A. bertolonii* ($p < 0.05$), and finally, 4.86 g plant^{-1} in *N. goesingensis* ($p < 0.05$). Increasing the compost rate from 2.5% to 5% tended to increase plant biomass production (although not significantly); but an increase in the compost dose from 5% to 10% did not lead to any further improvements in shoot DW. In fact, in the case of *A. pintodasilvae* (Barazón population) and *A. malacitanum* (both populations) there was a substantial decrease in shoot DW compared to the lower doses (Figure 5.1).

Effect of fertilisation and compost amendments on plant nutritive status

Table 5.3 shows the shoot macro- and micro-nutrient concentrations for each of the six hyperaccumulators. The shoot ionome was dependent on the plant (sub)species and the soil treatment. All the soil treatments increased shoot P concentrations, and this effect was generally statistically significant ($p < 0.05$). Compost addition led to a more marked increase in P concentrations than NPK fertilisation, and the highest shoot P concentrations were found in either the 5% or 10% compost rates (of depending on the plant (sub)species and/or population). Shoot P concentration in plants growing in untreated soils ranged from 0.36 g kg^{-1} in *N. goesingensis* to 0.99 g kg^{-1} in *A. pintodasilvae* (Samil) and increased up to 7.74 g kg^{-1} in *A. malacitanum* (S. Bermeja) in 5% compost (which corresponds with a 10-fold increase; $p < 0.05$). On the other hand, after inorganic fertilisation the maximum shoot P concentration reached was 2.89 g kg^{-1} in *A. pintodasilvae* (Barazón), representing an increase of up to 3-fold ($p < 0.05$). Shoot Ca concentrations varied markedly according to the treatment: NPK fertilisation decreased the shoot Ca concentrations of all six hyperaccumulators; while compost amendments increased Ca accumulation. This effect was stronger in those plants grown in the two higher addition rates (5% and 10%). Shoot Ca concentrations of untreated plants ranged from 10.9 g kg^{-1} in *N. goesingensis* to 37.4 g kg^{-1} in *A.*

Table 5.3. Shoot macro- and micro-nutrients concentrations in the six hyperaccumulators grown in untreated, NPK fertilised and compost-amended soils (mean concentration ± *SE*). Different letters indicate significant differences between treatments for each plant ($p < 0.05$; $n = 5$).

			P (g kg^{-1})	K (g kg^{-1})	Ca (g kg^{-1})	Mg (g kg^{-1})	Fe (g kg^{-1})
A.pintodasilvae	Barazón	Untreated	0.94 ± *0.08* c	11.7 ± *2.3* c	24.1 ± *2.0* b	19.1 ± *1.6* a	0.60 ± *0.07* a
		NPK	2.89 ± *0.15* b	37.4 ± *1.0* a	12.1 ± *0.3* c	21.3 ± *1.1* a	0.18 ± *0.02* b
		2.50%	4.83 ± *0.52* a	33.3 ± *3.8* ab	31.3 ± *2.3* b	12.4 ± *1.1* b	0.21 ± *0.02* b
		5%	6.40 ± *0.65* a	26.9 ± *1.5* b	43.9 ± *2.6* a	9.1 ± *0.8* b	0.24 ± *0.05* b
		10%	2.90 ± *0.39* b	13.6 ± *1.3* c	29.6 ± *3.7* b	22.9 ± *2 .4* a	0.27 ± *0.06* b
	Samil	Untreated	0.99 ± *0.03* c	17.1 ± *3.9* c	37.4 ± *6.9* bc	18.9 ± *1.9* a	0.86 ± *0.20* a
		NPK	2.35 ± *0.10* b	33.2 ±*1.1* a	12.4 ± *1.3* d	20.5 ± *0.4* a	0.36 ± *0.08* b
		2.50%	3.22 ± *0.11* b	35.3 ± *4.0* a	34.4 ± *0.9* c	12.4 ± *1.2* b	0.16 ± *0.02* b
		5%	4.31 ± *0.11* a	28.8 ± *1.9* bc	45.6 ± *1.2* ab	8.6 ± *0.6* b	0.27 ± *0.02* b
		10%	4.80 ± *0.33* a	27.2 ± *2.1* bc	49.4 ± *2.2* a	8.9 ± *0.7* b	0.25 ± *0.01* b
A. malacitanum	S. Bermeja	Untreated	0.74 ± *0.09* c	19.7 ± *1.4* a	27.0 ± *2.6* b	16.7 ± *0.8* ab	0.46 ± *0.07* a
		NPK	1.80 ± *0.29* c	27.8 ± *3.9* a	11.3 ± *0.9* c	20.9 ± *2.3* a	0.24 ± *0.05* b
		2.50%	3.62 ± *0.99* bc	27.7 ± *6.4* a	26.2 ± *6.0* b	8.7 ± *2.0* c	0.10 ± *0.02* b
		5%	7.74 ± *0.79* a	25.6 ± *1.8* a	40.6 ± *1.1* a	6.9 ± *0.4* c	0.11 ± *0.01* b
		10%	6.74 ± *1.34* ab	21.1 ± *2.2* a	43.9 ± *2.3* a	11.1 ± *2.0* bc	0.18 ± *0.01* b
	S. Aguas	Untreated	0.52 ± *0.10* d	22.0 ± *2.6* ab	16.6 ± *1.4* c	15.2 ± *0.6* ab	0.37 ± *0.05* a
		NPK	1.93 ± *0.09* cd	31.1 ± *4.0* a	9.3 ± *1.1* c	18.4 ± *0.8* a	0.29 ± *0.04* a
		2.50%	3.41 ± *0.13* bc	32.3 ± *2.1* a	28.2 ± *1.4* b	10.9 ± *0.6* bc	0.10 ± *0.01* b
		5%	5.07 ± *0.63* ab	28.1 ± *1.9* ab	43.0 ± *2.5* a	7.8 ± *0.7* c	0.10 ± *0.01* b
		10%	6.63 ± *1.22* a	19.0 ± *2.2* b	45.2 ± *3.8* a	13.3 ± *3.4* abc	0.11 ± *0.01* b
A. bertolonii		Untreated	0.67 ± *0.11* c	17.0 ± *1.7* a	14.3 ± *1.4* c	10.6 ± *1.5* b	0.68 ± *0.12* a
		NPK	2.44 ± *0.23* bc	22.3 ± *1.4* a	6.1 ± *0.2* c	16.1 ± *1.0* a	0.21 ± *0.02* b
		2.50%	4.42 ± *0.97* ab	26.5 ± *4.1* a	26.0 ± *3.9* b	10.7 ± *0.8* b	0.12 ± *0.01* b
		5%	5.40 ± *0.44* a	26.0 ± *2.1* a	33.6 ± *2.0* a	6.4 ± *0.5* c	0.12 ± *0.01* b
		10%	4.13 ± *0.19* ab	22.2 ± *1.3* a	37.0 ± *1.8* a	5.1 ± *0.3* c	0.12 ± *0.02* b
Noccaea goesingensis		Untreated	0.36 ± *0.02* c	10.2 ± *2.8* b	10.9 ± *1.8* c	13.0 ± *1.3* a	2.25 ± *0.58* a
		NPK	1.30 ± *0.13* c	17.5 ± *2.5* ab	2.8 ± *0.3* c	5.9 ± *0.7* b	0.22 ± *0.05* b
		2.50%	2.50 ± *0.29* b	17.3 ± *2.2* ab	12.8 ± *1.5* b	7.8 ± *0.9* b	0.17 ± *0.03* b
		5%	3.70 ± *0.29* a	26.1 ± *0.4* a	22.3 ± *2.2* ab	7.3 ± *0.9* ab	0.27 ± *0.03* b
		10%	3.06 ± *0.42* ab	23.7 ± *6.7* ab	22.5 ± *2.9* a	10.8 ± *2.4* ab	0.36 ± *0.08* b

pintodasilvae (Samil), while in the NPK fertilised soil they ranged from 2.8 g kg^{-1} in *N. goesingensis* to 12.4 g kg^{-1} in *A. pintodasilvae* (Samil) (representing a 3- to 4-fold decrease). On the other hand, the highest shoot Ca concentration were found in *A. pintodasilvae* (Samil) grown in the compost-amended soils: 49.4 g Ca kg^{-1} in the 10% compost and 45.6 g Ca kg^{-1} in the 5% compost treatment, respectively. Shoot K concentration in untreated plants ranged from 11.7 g kg^{-1} in *A. pintodasilvae* (Barazón) to 22.0 g kg^{-1} in *A. malacitanum* (S. Aguas). All soil treatments; both NPK fertilisation and compost amendment, increased shoot K concentrations. In general, the highest improvements were observed after NPK fertilisation (up to 37.4 g kg^{-1} in *A. pintodasilvae* (Samil)) and the 2.5% compost amendment rate (up to 35.3 g kg^{-1} in *A. pintodasilvae* (Samil)); while the highest compost rate had the least effect on shoot K accumulation. In contrast, all soil treatments led to a significant decrease in shoot Fe concentration and no differences were observed amongst treatments. Shoot Fe concentrations were considerably higher in *N. goesingensis* growing in untreated soils (2.25 g kg^{-1}) compared to the *Alyssum* hyperaccumulators (ranging from 0.37 g kg^{-1} to 0.86 g kg^{-1}). Variable effects on shoot Mg concentrations were observed, although some tendencies could be seen. The NPK treatment tended to increase Mg concentrations in plants (except *N. goesingensis*), although this was only significant for *A. bertolonii* ($p < 0.05$). On the contrary, compost treatments reduced Mg concentrations, and this effect was less pronounced in plants grown at the highest compost rate of 10% (except in *A. bertolonii*).

Effect of fertilisation and compost amendments on soil Ni availability, shoot Ni concentration, Ni yield and bioconcentration factor (BCF)

Soil Ni availability was assessed at the end of the experiment through $Sr(NO_3)_2$ extraction (Figure 5.2). After plant growth, in untreated soils available Ni ranged from 4.23 mg kg^{-1} in soils planted with *A. pintodasilvae* (Barazón) to 3.25 mg kg^{-1} in soils planted with *A. bertolonii.* Compared to untreated soil, a reduction in soil Ni availability was detected after plant growth in all the soil treatments, except in two cases with NPK fertilisation (Figure 5.2). Soil Ni availability was lowest in the 10% compost treatment, and especially after growth of *A. pintodasilvae* (Barazón); in this treatment $Sr(NO_3)_2$-extractable Ni concentrations were 0.08 mg kg^{-1} (53-fold lower than in untreated soils and 15-fold lower than in the 2.5% compost treatment; $p < 0.05$). In the NPK treatment, $Sr(NO_3)_2$-extractable Ni concentrations in pots planted with *A. pintodasilvae* (Samil)

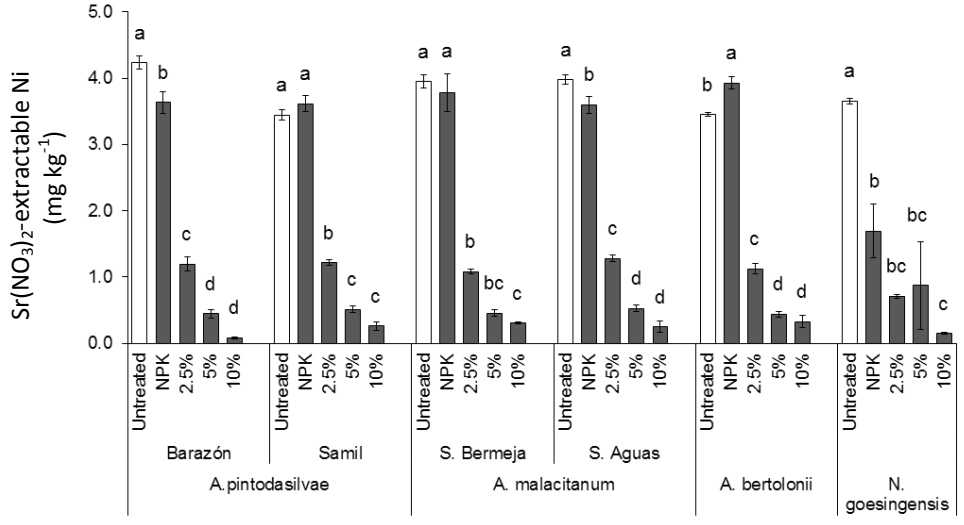


Figure 5.2. Effects of soil treatments (inorganic fertilisation (NPK) and organic amendment (addition rates of 2.5, 5 and 10%) on soil Ni availability assessed through $Sr(NO_3)_2$ extraction. Different letters above bars indicate significant differences between treatments ($p < 0.05$; $n = 5$).

increased from 3.44 mg kg^{-1} (untreated soil) to 3.62 mg kg^{-1} ($p > 0.05$) and in *A. bertolonii* available Ni was significantly increased (from 3.45 mg kg^{-1} to 3.93 mg kg^{-1}; $p < 0.05$).

In contrast to what was observed for plant biomass production, but in agreement with the soil Ni availability, compost treatments significantly reduced shoot Ni concentrations compared to plants growing in untreated soils (Figure 5.3a) On the other hand, the NPK treatment had no pronounced effect on shoot Ni accumulation although led to a significant reduction in the shoot Ni concentration in *A. malacitanum* (S. Bermeja) and *N. goesingensis* ($p < 0.05$), while a slight increase (but not significant) was observed in *A. pintodasilvae* (Samil). In contrast, the reduction in shoot Ni concentrations of plants growing in compost-amended soils was consistently observed in all six hyperaccumulators and at all threes rates of addition ($p< 0.05$). Moreover, an increase in the compost rate (from 5% to 10%) was accompanied by further declines in shoot Ni concentration (Figure 5.3a). The maximum decrease in shoot Ni concentration after addition of 2.5% compost was 4-fold (this was recorded for the S. Bermeja population of *A. malacitanum*, decreasing from 9528 mg Ni kg^{-1} in untreated soil to 2584 mg Ni kg^{-1} in 2.5% compost). While a 29-fold decrease in shoot Ni concentration was observed in *A. pintodasilvae* (Barazón) after 10% compost addition (decreasing from 5714 mg Ni kg^{-1} in untreated soil to 197 mg Ni kg^{-1} in 10% compost).

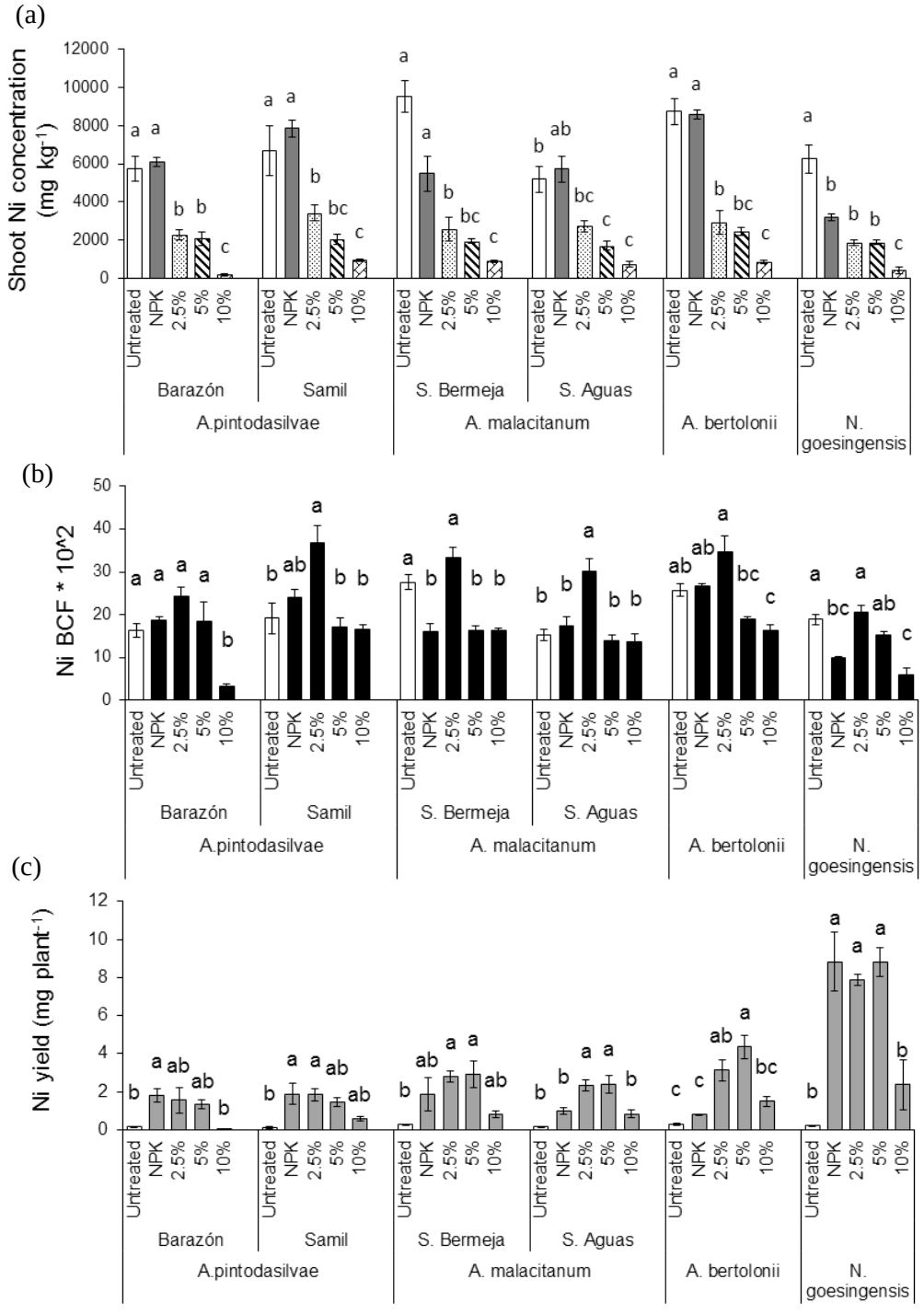


Figure 5.3. Effects of soil treatments (inorganic fertilisation (NPK) and organic amendment (addition rates of 2.5, 5 and 10%) on (a) shoot Ni concentration, (b) Ni BCF and (c) Ni yield. Different letters above bars indicate significant differences between treatments ($p < 0.05$; $n = 5$).

Contrary to what was observed with Ni availability, compost treatment did not generally lead to a decrease in the Ni BCF and, in some cases, even increased it (for example in the 2.5% compost treatment; Figure 5.3b). This increase in the Ni BCF in the low compost treatment was significant for *A. pintodasivae* (Samil) (36.7×10^2; $p < 0.05$). On the other hand, a significant reduction in Ni BCF was generally only observed at the highest compost treatment (10%): the lowest BCF values were found in *A. pintodasilvae* (Barazón) (3.2×10^2) and in *N. goesingensis* (5.9×10^2). The 5% compost treatment and NPK fertilisationsignificantly decreased Ni BCF in *A. malacitanum* (S. Bermeja) and *N. goesingensis* (only for NPK) ($p < 0.05$).

Compared to untreated soils, all the soil treatments (with the exception of 10% compost in *A. pintodasilvae* (Barazón)) increased the amount of phytoextracted Ni (Ni yield) (Figure 5.3c). The highest beneficial effects of soil treatments on plant Ni yield were generally obtained in the 2.5% and 5% compost treatments. Moreover, these positive effects were more marked in *N. goesingensis* (in this plant species both the NPK treatment and compost additions caused a strong increase in Ni yield). Ni removal by *N. goesingensis* was 8.8 mg Ni plant^{-1} in NPK fertilised soil, 7.9 mg plant^{-1} in 2.5% compost, and 8.8 mg plant^{-1} in 5% compost. However, its efficiency of Ni phytoextraction was significantly reduced at the higher compost (10%) addition (2.4 mg Ni plant^{-1}; $p < 0.05$). For *Alyssum*, plant Ni yields in compost-amended soils were similar amongst the two *A. serpyllifolium* subspecies and their populations (ranging from 1.6 to 2.8 mg Ni plant^{-1} in 2.5%, from 1.3 to 2.9 mg Ni plant^{-1} in 5% and from 0.05 to 0.8 mg Ni plant^{-1} in 10%), while *A. bertolonii* achieved a higher Ni yield in the compost-amended soils (3.1 mg Ni plant^{-1}, 4.4 mg Ni plant^{-1} and 1.5 mg Ni plant^{-1} in 2.5%, 5% and 10% compost respectively). For *Alyssum*, the highest benefits of the NPK treatment on Ni yield were observed in both populations of *A. pintodasilvae* (1.8 mg Ni plant^{-1} for the Barazón population and 1.9 mg Ni plant^{-1} for Samil) and the S. Bermeja population of *A. malacitanum* (1.9 mg Ni plant^{-1}).

5.4. Discussion

The six hyperaccumulator plants showed a similar growth in the untreated soil. The Barazón population of *A. pintodasilvae* did not show better growth despite the fact that this subspecies is native to the site where the soil was collected. Plant growth was extremely poor in the untreated soil, and much lower (approximately 10-fold) than what was expected in comparison to previous

studies where *A. pintodasilvae* was grown in the same soil (Cabello-Conejo *et al.*, 2014a). Nonetheless, all six hyperaccumulators presented shoot Ni concentrations which were well above the threshold value defined for Ni hyperaccumulation (1000 mg kg^{-1}). In the case of the S. Bermeja population of *A. malacitanum* plants obtained close to 1% DW of Ni in their aboveground tissues. However, limited growth reduced the corresponding amount of phytoextracted Ni (in untreated soils less than 0.1% of the total Ni in soil was removed by plants (data not shown)). In the case of *Alyssum*, differences were observed in shoot Ni accumulation between species, subspecies and populations of subspecies. *Alyssum malacitanum* (only the S. Bermeja population) and *A. bertolonii* were able to obtain higher shoot concentrations of Ni compared to *A. pintodasilvae*.

Serpentine soils typically present unfavourable conditions for plant growth, such as a deficiency in essential nutrients (such as N, P and K) and organic matter. Maximising the performance and yield of hyperaccumulator crops is therefore essential in the development of phytomining systems. This study showed that inorganic fertilisation could improve soil fertility and hence plant growth, and confirmed the existence of nutritional deficiency in serpentine soils. A significant increase in plant DW yield, and shoot K and P concentrations, was observed in all six hyperaccumulators in NPK-treated soils. These essential nutrients are known to be low in ultramafic soils and yet fairly accumulated by most hyperaccumulator plants (Zhang *et al.*, 2014). These results support previous studies which have also shown stimulation in the biomass production of *Alyssum* species after the addition of inorganic fertilisers (Robinson *et al.*, 1997; Chaney *et al.*, 2008; Cabello-Conejo *et al.*, 2014b). Cabello-Conejo *et al.* (2014b) obtained a similar DW yield in the SB population of *A. malacitanum* as that obtained in this study: this subspecies was grown in the same serpentine soil and with the same NPK fertilisation as used here (growth time was 4 months compared to 5 months here). Inorganic fertilisation consisting in 100:150:200 kg ha^{-1} NPK was also shown to effectively increase DW yields (10-fold) of *A. pintodasilvae* in contaminated mine-soils (Kidd and Monterroso, 2005). Moreover, mineral fertilisation (120 kg NPK ha^{-1} and 77 kg Ca ha^{-1}) has been successfully used on a field basis to improve the biomass production of *Alyssum murale*, obtaining yields of up to 9.0 t ha^{-1} (Bani *et al.*, 2015).

In general, compost amendment had an even higher growth-promoting effect than inorganic fertilisation. Composts can not only provide a source of essential nutrients (which are limiting in serpentine soils) but also improve soil

structure and porosity, and water holding capacity, which could explain the more pronounced effects of compost additions that were observed on plant yields. The addition of composted MSW improved soil physicochemical properties, such as nutrient availability, CEC, or organic matter content. The compost used in this study also significantly improved the soil Ca/Mg ratio. Shoot Ca concentrations were often reduced in plants grown in NPK-treated compared to untreated soils, whereas compost additions led to a significant improvement in the shoot Ca concentration. This was particularly the case at the 5 and 10% addition rates. Serpentine soils characteristically present a low Ca concentration (and unfavourable Ca/Mg ratio for plant growth) and Ni hyperaccumulators take up large amounts of Ca from them, hence the repeated harvest and removal of biomass during phytomining processes requires the inclusion of Ca fertilisation to maintain fertility at appropriate levels (Kukier *et al.*, 2004; Chaney *et al.*, 2007; Chaney *et al.*, 2008). Other authors have added gypsum to serpentine soils to overcome this limitation (Cabello-Conejo *et al.*, 2013), but it seems that organic amendments (such as the composts used here) could also fulfil this plant requirement. Plants grown in compost-amended soils also presented a higher shoot P and K concentration than plants grown in untreated soil. On the other hand, shoot Fe and Mg concentrations were reduced (in both compost- and NPK-treated soils) but neither element reached deficiency levels (50-150 mg kg^{-1} for Fe, 1.5-3.5 g kg^{-1} for Mg, according to Marschner (1995)).

The Ca/Mg ratio has been shown to be a relatively important factor for growth and Ni uptake of hyperaccumulators. Ghasemi and Ghaderian (2009) studied the effect of the Ca/Mg quotient in two populations (serpentine and non-serpentine) of the hyperaccumulator *A. inflatum* and found that the serpentine population showed a higher tolerance to a low Ca/Mg ratio. The same authors also observed an increase in plant Ni accumulation with increasing Ca/Mg ratio in hydroponic solutions. However, in the present study, a decrease in shoot Ni concentration was found alongside the increase in the soil Ca/Mg ratio produced in compost-amended soils. Similarly, Brooks *et al.* (1981) showed that *A. serpyllifolium* was able to tolerate higher Ni concentrations in the presence of high concentrations of Ca in the soil by lowering its Ni uptake. Thus when using compost amendments it will be important to manipulate the soil Ca/Mg ratio in order to obtain an optimum balance between plant growth and Ni accumulation in phytomining systems.

The use of organic amendments based on waste products has rarely been

considered for stimulating the growth of hyperaccumulating plants in serpentine soils. Here, the stimulation in plant growth was observed in hyperaccumulating plants belonging to two distinct genera (*Alyssum* and *Noccaea*). In fact, *N. goesingensis* responded most to the different soil treatments in terms of biomass production (and consequently Ni removal). The serpentine site of Redlschlag where *N. goesingensis* is endemic presents soil physicochemical characteristics which are likely to be more favourable for plant growth than the serpentine site of Barazón where the soil in this study was collected (unpublished data). The adaptation of this species to the soil conditions in its natural habitat could explain the stronger positive response observed in its growth after improvements in soil fertility. Ni-hyperaccumulating subspecies of *Alyssum serpyllifolium* have often been suggested to present a limited application potential in phytomining systems compared to other *Alyssum* sp., such as *A. murale* or *A. corsicum*, due to their low productivity (Cabello-Conejo *et al.*, 2013). However, the shoot biomass of *A. malacitanum* (both populations) obtained in the soils with 5% w/w compost was close to that of *A. bertolonii*. Moreover, the stimulation in biomass produced through compost additions was 4-fold higher than that obtained using phytohormone applications by Cabello-Conejo *et al.* (2014b). These authors obtained a mean shoot DW yield in *A. murale* (grown in the same serpentine soil as used here) which was of a similar magnitude to that obtained in *A. malacitanum* with 5% w/w compost addition.

The beneficial effects of compost addition were no longer observed at the highest rate of addition (10% w/w). Other authors have found a similar decrease in plant yields of agricultural crops with an increase in the rate of addition of compost to metal-contaminated soils. Alvarenga *et al.* (2009) found that increasing compost (based on municipal residues) addition from 50 Mg ha^{-1} to 100 Mg ha^{-1} to a mine-soil did not induce a higher biomass production of perennial ryegrass. These authors attributed this to an increase in soil salinity due to the addition of soluble salts with the compost. In this study, a possible explanation for the decrease in the yields of *Alyssum* could be the addition of potentially toxic metals to the soil (other than Co, Cr or Ni) together with the compost. The pseudo-total soil Cu concentration was 7-fold higher in the 10% compost-amended soil (77.8 mg kg^{-1}) compared to the untreated soil (11.0 mg kg^{-1}). Copper has previously been shown to be phytotoxic to *A. pintodasilvae* at high concentrations (Kidd and Monterroso, 2005), and minimal Cu tolerance was observed in a range of Ni-hyperaccumulators within the *Alyssum* genus (Ghasemi

et al., 2015). Other studies suggest that the decrease in soil Ni availability could also negatively affect the growth of hyperaccumulators. For example, Sellami *et al.* (2012) found the growth of *A. murale* was enhanced in soils amended with industrial sewage sludge with increasing Ni concentration compared to the non-amended control, which could indicate the high Ni requirement of this plant. In the present study, the reduced growth observed at 10% compost addition could reflect a similar Ni requirement (also reflected in the significant decrease in Ni BCF at the highest dose of compost in four of the Ni hyperaccumulators). Our results therefore show that compost addition can increase soil organic matter content and fertility of serpentine soils, however, it will be important to optimise the rates of addition to achieve optimal plant growth and metal extraction.

Soil Ni availability differed between soil treatments, and was dependent on the type of treatment (inorganic vs. organic fertilisation) and also on the rate of compost addition (in the case of organic amendment). Before plant growth, Ni availability was not modified by NPK fertilisation compared to untreated soil. Similarly, Robinson *et al.* (1997) found no differences in soil Ni extractability after incorporating inorganic fertilisers (NH_4NO_3, K_2SO_4, $Ca(H_2PO_4)_2$) in a serpentine soil where *A. bertolonii* was grown. In contrast to NPK fertilisation, compost addition led to a strong reduction in soil Ni availability. This could partly, but not entirely, be explained by a soil dilution effect; after compost addition a decrease of up to 1.6-fold was recorded in the pseudo-total Ni concentration after 10% w/w compost addition. However, the corresponding decrease in the NH_4Cl-extractable Ni concentration (11-fold) indicates that processes other than simple soil dilution were influencing metal mobility. Organic soil amendments have frequently been shown to decrease soil metal bioavailability through the formation of organo-metallic insoluble complexes (Mench *et al.*, 2000; Vangronsveld *et al.*, 2009). The composting of organic residues also reduces the dissolved organic carbon thus stabilising the wastes and decreasing metal mobility (Kiikkilä *et al.*, 2002). The effect of compost addition on soil Ni availability reinforces the need for optimising the rate of addition to reach maximal biomass productivity but minimise reductions in plant-available metal fractions and thus metal uptake and accumulation.

After plant growth, soil Ni availability was consistently lower than in untreated soil in all compost-amended treatments and for all plant species. In the 10% compost treatment, the $Sr(NO_3)_2$-extractable concentration was reduced by up to 7-fold compared to before plant growth, which could again suggest Ni was

limiting plant growth in this treatment. On the other hand, plant growth modified soil Ni availability in the NPK treatment, and this was dependent on the plant species. In general, a slight decrease in $Sr(NO_3)_2$–extractable Ni concentrations compared to untreated soil was found in those pots cultivated with *A. pintodasilvae* and *A. malacitanum*. In the case of *N. goesingensis* a similar decrease was observed but much more pronounced (more than 2-fold), which could not be attributed to a more prolific plant growth and Ni uptake. Finally, after growth of *A. bertolonii* a significant increase in soil Ni availability was observed. In addition, to this plant species effect, the type and composition of inorganic fertilisers could be selected in order to enhance metal bioavailability and therefore the efficiency of phytoextraction (Kidd *et al.*, 2015). For example, acidification induced by ammonium-fed plants can promote mobilisation of metals in soils (Kidd *et al.*, 2015). However, Schwartz *et al.* (2003) showed that at higher N additions, NO_3^- significantly increased Cd uptake by *Noccaea caerulescens* compared to NH_4^+. This effect would be consistent with other studies showing an increase in Ni uptake of hyperaccumulators with increasing soil pH, thus indicating that the hyperaccumulation process does not seem to be related to soil acidification (Li *et al.*, 2003b). It would be interesting to test different fertilisation regimes for achieving an optimal balance between plant biomass stimulation, enhanced soil metal availability and hence plant uptake and accumulation. Additionally, in long-term phytomining strategies if biomass production is to be maintained over several years, it will be necessary to adjust the fertiliser application so as to replace exported nutrients with each harvest.

No positive effects on shoot Ni concentrations were found after either NPK or compost treatment. In fact, after NPK fertilisation shoot Ni concentrations were significantly reduced in *N. goesingensis* (but not *Alyssum* (sub)species) compared to plants grown in untreated soil. In agreement, Bani *et al.* (2015) found that soil fertilisation did not influence Ni uptake by *A. murale*. Some authors have observed a decrease in shoot Ni concentrations alongside the stimulation in biomass production; for example, both Robinson *et al.* (1997) and Kidd and Monterroso (2005) observed a slight, but significant, decrease in Ni uptake of *A. bertolonii* and *A. pintodasilvae* after fertilisation. However, these authors observed that Ni yield was still increased due to the promotion in plant biomass production. The same was true in this study, where phytoextracted Ni was increased after NPK addition due to the fertiliser-induced promotion in growth. A significant and negative effect on shoot Ni concentrations was also observed after

compost amendment, and this was found for all rates of addition (in line with the observed reductions in soil Ni availability). However, the pronounced positive effect on plant biomass production significantly increased phytoextracted Ni. Moreover, since compost had a more beneficial effect on plant yield than NPK fertilisation, the overall phytoextracted Ni was also higher in these treatments. Most literature related to the use of organic amendments in soil phytoremediation focuses on (aided) phytostabilisation, while few studies have evaluated their use in metal phytoextraction using metal-accumulating plant species, and rarely with hyperaccumulators. Fiorentino *et al.* (2013) improved the growth of *Arundo donax* growing in a Cd-contaminated soil after compost application at the same time than an increase in Cd, Cu and Cr uptake. Pachura *et al.* (2015), observed an increase in Cd uptake after addition of compost based on sewage sludge and forest waste by *Virginsa fanpetals* growing in a Cd- and Zn- contaminated soil. Schwartz *et al.* (2003) reported increases in Cd uptake, biomass production, and Zn/Cd yields of *N. caerulescens* after addition of non-contaminated sludge to a co -contaminated soil with inorganic and organic pollutants. To the best of our knowledge this is the first study to demonstrate the potential use of organic waste products as a means of improving Ni phytoextraction by Ni-hyperaccumulators. Field-based studies will be required to evaluate the long-term effects and potential benefits of compost amendments in phytomining systems. The need for additional compost dressings over time will also have to be considered.

5.5. Conclusions

In conclusion, both types of soil treatment (inorganic fertilisation and organic amendments) improved the total amount of Ni phytoextracted, and this was mainly through stimulation in biomass production and not through an improved Ni uptake by the hyperaccumulating plants. All the plant species accumulated Ni concentrations characteristic of hyperaccumulators, however the most efficient plants in terms of Ni removal were *N. goesingensis, A. bertolonii* and the SB population of *A. malacitanum*. In these plants, the best soil treatments were 2.5 or 5.0% w/w compost addition (and also the NPK treatment in the case of *N. goesingensis*). The strong reductions in soil Ni availability (and corresponding plant Ni accumulation) observed after compost addition highlight the need for further optimisation regarding the final rates of addition. Full deployment of phytomining systems at field scale will require agronomic practices which aim to maximise biomass production and Ni yield of hyperaccumulators.

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Chapter 6

Evaluating phytoextraction efficiency of two high-biomass crops after soil amendment and inoculation with rhizobacterial strains

ABSTRACT

We evaluated the effect of compost amendment and/or bacterial inoculants on the growth and metal accumulation of *Salix caprea* (clone BOKU 01 AT-004) and *Nicotiana tabacum* (*in vitro*-bred clone NBCu10-8). Soil was collected from an abandoned Pb/Zn-mine and rhizobacterial inoculants were previously isolated from plants growing in the same mine. Plants were grown in untreated or compost-amended (5% w/w) soil, and were inoculated with five rhizobacterial strains. Non-inoculated plants were also established as a control. Compost addition increased the shoot DW yield of *N. tabacum* but not *S. caprea*, while it decreased soil metal availability and lowered shoot Cd/Zn concentrations in tobacco plants. Compost amendment enhanced the shoot Cd/Zn removal due to the growth promotion of *N. tabacum* or to the increase in metal concentration in *S. caprea* leaves. Bacterial inoculants increased photosynthetic efficiency (particularly in *N. tabacum*), and sometimes modified soil metal availability, but this did not lead to a significant increase in Cd/Zn removal. Compost amendment was more effective in improving the Cd and Zn phytoextraction efficiency than bioaugmentation.

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6.1. Introduction

Mining activities and mineral processing are one of the main sources of anthropogenic metallic contamination worldwide (Panagos *et al.,* 2013), generating large volumes of waste materials with low contents of organic matter and essential nutrients, limited water holding capacity, often a high acidity and potentially toxic concentrations of trace elements (TE) (Wong, 2003). Gentle remediation options have been developed to (phyto)manage contaminated soils, aimed at decreasing the bioavailable TE pool and/or total TE contents in the soil through uptake and accumulation in plant biomass (e.g. phytoextraction), or promoting their *in situ* inactivation by use of TE-excluding plants and soil amendments (e.g. phytostabilisation) (Vangronsveld *et al.,* 2009; Mench *et al.,* 2010; Kidd *et al.,* 2015). High biomass species which are able to tolerate and accumulate TE in their harvestable parts have been suggested as good candidates for phytoextraction techniques. Within the Salicaceae family several species have been described as metal accumulators and show relevant shoot TE removals (i.e. moderate-high bioconcentration factor (BCF) and high shoot yield), many of these belong to the genus *Salix* (Dickinson and Pulford, 2005; Dos Santos Utmazian and Wenzel, 2007). Clones of *S. caprea* growing in contaminated soils have been shown to accumulate up to 116 mg Cd kg^{-1} and up to 4680 mg Zn kg^{-1} in their leaves (Unterbrunner *et al.,* 2007). *Salix* have a high aerial biomass production (up to 10–15 dry t ha^{-1} $year^{-1}$, (Riddell-Black *et al.,* 1997)) which is also easily harvestable, and an extensive root system which is able to explore a large soil volume (Pulford and Watson, 2003). In addition to woody species, high biomass and rapidly growing annual crop species have been incorporated into phytoextraction systems with promising results. Some varieties of *Nicotiana tabacum* were described as efficient Cd-accumulators in the early 80s (Isermann, 1983; Davis, 1984). More recently, tobacco *in vitro* cultivars have been bred showing enhanced metal-extraction for Cd, Pb and Zn (Guadagnini, 2000; Herzig *et al.,* 2014).

In addition to plant species selection, several authors have proposed engineering the plant-microbial-soil system as a means of enhancing the efficiency of the phytoextraction process (Kidd *et al.* (2015) and references therein). The application of organic amendments (such as composts) has been extensively used because of their stimulating effect on soil fertility and plant growth in contaminated soils (Kumpiene *et al.,* 2012; Martínez-Fernández *et al.,* 2014; Parraga-Aguado *et al.,* 2015). Alternatively, bacterial inoculants based on plant

growth promoting bacteria (PGPBs) can stimulate growth by improving plant nutritive status due to the presence of N_2 fixers, PO_4-solubilisers, or siderophore-producers, or by producing phytohormones (such as indoleacetic acid, IAA) or suppressing stress ethylene production (due to ACC deaminase activity) (Glick, 2010; Sessitsch *et al.*, 2013).

Both organic amendments and PGPB can influence soil metal availability. The addition of composts or biosolids has contrasting effects, varying according to their type, addition rate and metal in question. Although most studies associate their incorporation with metal immobilisation through the formation of insoluble organo-metal complexes (Mench *et al.*, 2000; Kumpiene *et al.*, 2008), others have found significant increases in metal mobility. Boyter *et al.* (2009) showed that Mn, Cu and Cd removal using willows was higher after addition of compost due to an increase in plant metal uptake. Plant-associated bacteria can increase TE availability through the release of chelating compounds or changes in pH or Eh (Kidd *et al.*, 2009; Sessitsch *et al.*, 2013). Becerra-Castro *et al.* (2011) found an increase in Ni solubilisation after bacterial inoculation of metal-enriched substrates, which was correlated with the release of oxalate or siderophores. Similar increases have been shown to promote metal accumulation and removal by (hyper)accumulating plants (Kuffner *et al.*, 2008; Becerra-Castro *et al.*, 2011; Cabello-Conejo *et al.*, 2014).

Few studies have evaluated the effects of combining both treatments: the use of organic amendments together with bacterial inoculants. Moreover, the beneficial effect of the inoculant could reduce the reliance on high compost rates required for plant establishment, thus reducing the costs of the remediation process. In this study we evaluate the metal phytoextraction efficiency of two high-yielding crops: *Salix caprea* L. and *Nicotiana tabacum* L. and the potential improvement with soil organic amendment and/or bioaugmentation. The specific objectives were to evaluate (i) the ability of the plant species to grow and accumulate TE when cultivated in a mine-soil contaminated with Cd, Pb and Zn, (ii) improvements in soil properties, plant growth and physiological status, and plant metal accumulation after compost addition to the mine-soil, and (iii) the use of PGPB for improving plant metal extraction alone or in combination with compost.

6.2. Materials and Methods

Mine-soil, plant species and rhizobacterial strains selected for the pot experiment

Salix caprea and *N. tabacum* were grown on a mine tailings soil collected from the abandoned Pb-Zn mine of Rubiais (NW Spain). Information on the mining history of the area, metal deposits and soil physicochemical properties is summarised in Monterroso *et al.* (2014). Briefly, the mine-soils present a large degree of heterogeneity and generally show unfavourable conditions for plant growth: very low nutrient availability, low cation exchange capacity, limited organic matter content and elevated concentrations of trace metals. The texture is a sandy loam-loam type and they have a high content in carbonates (10%). They present high concentrations of Cd, Pb and Zn bound to non-residual geochemical fractions (principally carbonates, Fe/Mn oxy(hydr)oxides and sulphides) (Monterroso *et al.*, 2014).

Soils were either untreated or amended with 5% (w/w) compost. The rate of addition was based on previous studies (unpublished results). After compost incorporation the soils were adjusted to 70% of their water holding capacity and left for 2 weeks to attain equilibrium before planting. The compost was provided by a local SME company dedicated to the recycling of waste products (Tratamientos Ecológicos de Noroeste, TEN s.l.) and is primarily based on municipal sewage sludge and wood chippings. The untreated and compost-amended soils, and the compost itself, were analysed for general physicochemical properties. Soil pH was measured in H_2O and 0.1 M KCl using a 1:2.5 soil:solution ratio. Total C and N were analysed by combustion with a CHN analyser (Model CHN-1000, LECO Corp., St Joseph, MI). Exchangeable cations were extracted with 1M NH_4Cl (1:20 soil:solution ratio) after 16 hours of equilibration and cationic exchangeable capacity (CEC) was calculated as the sum of exchangeable Ca, Mg, K, and Al. Soils (0.5 g) were digested in a 3:1 mixture of concentrated HNO_3:HCl after the addition of 3mL H_2O_2 in Teflon PFA vessels in a microwave accelerated reaction system (MarsXpress; CEM Corp., USA) and the total concentrations of metals were analysed by ICP-OES (Varian Vista Pro, Australia).

Salix caprea Mauerbach (BOKU 01 AT-004) was selected on the basis of previous studies for its Cd and Zn accumulation capacity (Dos Santos Utmazian *et al.*, 2007a; Dos Santos Utmazian and Wenzel 2007; Wieshammer *et al.*, 2007). The same clone showed a positive response in metal accumulation or biomass pro-

duction after inoculation with *Streptomyces* AR17 or *Agromyces* AR33 (Kuffner *et al.*, 2008). The *Nicotiana tabacum* variant NBCu10-8 was obtained through *in vitro* cultivation from metal-tolerant mother lines (Guadagnini, 2000; Herzig *et al.*, 2003). This variant showed shoot accumulation of Cd and Zn of up to 2-fold higher than that of the mother line (Herzig *et al.*, 2003; Lyubenova *et al.*, 2009).

The rhizobacterial strains used (P87, P29, P30, P64, P75) were previously isolated from the rhizosphere soils of pioneer plants growing at the same site where the mine-soil was collected (Becerra-Castro *et al.*, 2012). The strains were selected according to their MIC (minimum metal inhibitory concentration) values and phenotypic traits (Table 6.1). All of the selected strains were found to increase the biomass of either *Salix viminalis* or *Festuca arundinacea*, or both, in previous inoculation trials (Becerra-Castro *et al.*, 2012).

Table 6.1. Phenotypic characteristics of the 5 rhizobacterial strains selected for the inoculation experiment

Host	Isolate	Strain	MIC		PO_4	Surf	Sid	IAA (mg L^{-1})
			Cd (mM)	Zn (mM)				
Festuca	P87	*Massilia niastensis*	0.5	1	-	-	-	9.03
Festuca	P29	*Pseudomonas costantinii*	0.5	0.5	+	+	-	-
Festuca	P30	*Rhodococcus erythropolis*	4	2.5	-	-	-	4.97
Cytisus	P64	*Streptomyces coelescens*	5	5	-	-	+	-
Festuca	P75	*Tsukamurella strandjordii*	5	25	-	+	-	-

MIC, minimum inhibitory concentration (indicative metal tolerance); PO_4, Phosphate solubilisation; Surf, surfactant production; Sid, Siderophore production; IAA, indoleacetic acid production (previously characterised by Becerra-Castro *et al.* (2012))

Experimental set-up and inoculation of plants

Tobacco seeds were germinated in commercial potting mixture and willow cuttings (approximately 20 cm in length) were rooted in sand. One-month-old tobacco seedlings and 6-month-old rooted willow cuttings were transplanted into pots containing 3 kg of either untreated or compost-amended mine-soil. Plants were left for one month and after this adaptation period bacterial inoculation was carried out. Fresh cultures of bacterial strains were grown in 869 liquid medium

(Mergeay *et al.*, 1985) for 24 h, harvested by centrifugation (6000 rpm, 15 min) and re-suspended in 10mM $MgSO_4$ to an OD_{660} of 1.0 (about 10^7 cells per mL). Each plant pot was inoculated with 10 mL of bacterial suspension. The same amount of sterile 10mM $MgSO_4$ was added to non-inoculated pots. Five replicates of each plant species were prepared for each inoculation treatment and soil type. Plants were watered regularly to maintain soil moisture and grown under a natural day/night light cycle in greenhouse conditions.

Chlorophyll fluorescence

Chlorophyll fluorescence parameters were measured before harvesting with a portable pulse amplitude-modulated fluorometer (MINI-PAM photosynthesis yield analyser; Walz, Effeltrich, Germany) by the saturation pulse method (Schreiber *et al.*, 1998). Measurements were taken on the upper surface of a fully expanded leaf on each plant. The maximum quantum yield of photo-system II (PSII) was recorded, which was assessed by the ratio $F_v/F_m = (F_m - F_0)/F_m$ (Bolhar-Nordenkampf *et al.*, 1989), where F_0 and F_m are the minimal and maximal fluorescence yields of a dark-adapted sample, respectively, with all PSII reaction centres fully open (i.e. all primary acceptors oxidised). This parameter was measured after a 30-min period of dark adaptation, time enough to ensure that all their PSII reaction centres were open. F_v/F_m characterises the photosynthetic process associated with electron transport (light reactions), estimates the efficiency of excitation energy capture by open PSII reaction centres (Butler and Kitajima, 1975), and is correlated with the amount of carbon gained per unit of light absorbed (Bolhàr-Nordenkampf and Öquist, 1993).

Leaf spectral reflectance

Immediately after the chlorophyll fluorescence measurements and on the same leaves, spectral reflectance parameters were determined using a portable spectrometer (UniSpec Spectral Analysis System, PP Systems, Haverhill, MA). Reflectance indices were measured as follows: (1) the photochemical reflectance index (PRI) was calculated as (R531 - R570) / (R531 + R570), where R531 and R570 are reflectance at 531 and 570 nm, respectively. Previous studies have demonstrated that PRI is significantly correlated with both net CO_2 uptake and the photosynthetic radiation-use-efficiency (mol CO_2 mol^{-1} photons) (Peñuelas *et al.*, 1995). PRI has also been correlated inversely with the levels of de-epoxidised (photoprotective) xanthophyll cycle pigments (Sims and Gamon, 2002). (2) The

chlorophyll content index (CHL) was calculated as R750/R700, where R750 and R700 are reflectance at 750 and 700 nm, respectively. This index is correlated closely with the chlorophyll content of leaves (Lichtenthaler *et al.*, 1996; Richardson *et al.*, 2002).

Plant and soil analyses

After a 3 month growth period, plants were harvested and shoot dry weight (DW) yield determined. Leaves, stems and roots of plants were separated, washed in deionised water, oven-dried at 45 °C, weighed and ground. Oven-dried leaves and stems were digested in a 2:1 HNO_3:HCl mixture and the concentrations of P, K, Ca, Mg, Fe, Cd, Pb and Zn were measured by ICP-OES. After plant growth, metal availability was evaluated by extraction with 1 M NH_4NO_3 (1:2.5 w/v, 2 h shaking) and Cd, Pb and Zn were analysed by atomic absorption spectroscopy (AAS; Perkin-Elmer 2380, Norwalk, CT). Metal fractionation was assessed following a modified version of the three step BCR sequential extraction procedure (Rauret *et al.*, 2000). The BCR procedure was modified to separate exchangeable metals (extracted with 1 M NH_4Cl, 16 h equilibrium) and carbonate -bound metals (extracted with 0.11 M CH_3COOH, 16 h shaking), due to the importance of this latter phase in this soil. Five soil fractions were obtained: F1 corresponds with the water-soluble and exchangeable metal fraction (exchangeable), F2 with the acid-extractable metal fraction (carbonate-bound), F3 the reducible fraction (iron and manganese oxide bound forms), F4 the oxidisable fraction (organically bound and sulphide metals) and finally, F5 represents the residual fraction (silicate-bound metals). Five replicates were used for all soil analyses (including the metal fractionation) and plant analyses.

The BCF was calculated as the concentration of metal in plant tissues divided by the initial exchangeable fraction of metal in soils (using the NH_4Cl-extractable metal concentration). Since phytoextraction aims to reduce the labile fraction of metals in the soil ("*bioavailable stripping*") we considered the available metal fraction (in this case the NH_4Cl-extractable concentration) to give a better estimation to the plant's ability to concentrate metals in the aerial tissues. The phytoextracted metal (or metal yield) was calculated on the basis of shoot biomass production and shoot metal concentration.

Statistical analyses

Differences between plant species and soil treatments were determined using a Student's t-test for independent means. Effects of bacterial inoculation on plant biomass production, physiological measurements, shoot metal and nutrient concentrations, and soil metal concentrations were determined using analyses of variance (ANOVA). A multiple comparison of means was determined by the "post -hoc" Dunnet test. When necessary, data were transformed to meet the requirements of ANOVA. Data that were not normally distributed were analysed using the Kruskal-Wallis non parametric tests.

6.3. Results

Effect of compost on soil properties and metal availability

The general properties of the untreated and compost-amended soils are presented in Table 6.2. The mine-soil presented a neutral pH with a low total C and N content (1.50% and 0.07% respectively), and low available P (6.6 mg kg^{-1}). Cation exchange capacity (CEC) was high (31.3 $cmol_c$ kg^{-1}) and dominated by Ca (representing >95% of exchangeable cations). Total concentrations of Cd, Zn and Pb were 6 mg kg^{-1}, 3398 mg kg^{-1} and 234 mg kg^{-1}, respectively. NH_4Cl-extractable Zn presented the highest concentrations (316 mg kg^{-1}), followed by Cd (4.4 mg kg^{-1}) and Pb (2.2 mg kg^{-1}). The compost presented a pH close to neutrality, C/N ratio of 13.5, high CEC (57.6 $cmol_c$ kg^{-1}), and a low to moderate (in the case of Cu) concentration of trace metals. No changes in pH were found after compost addition to the mine-soil, while the amount of C and N were 1.7- and 1.4-fold higher, respectively, compared to the untreated soil. Available P was also increased by 18%, while CEC was reduced by 44% (from 31.3 $cmol_c$ kg^{-1} to 17.6 $cmol_c$ kg^{-1}). However, the main effect of the compost addition was a pronounced decrease in exchangeable Ca (up to 2-fold), while the other exchangeable cations increased (K, Mg and Na). Finally, addition of compost led to a 2-fold decrease in Zn, Cd and Pb availability in amended soils (NH_4Cl-extractable concentrations).

Table 6.2. Physicochemical characteristics of the untreated and compost-amended soils and of the compost used for amending.

	Untreated soil			Compost-amended soil			Compost amendment			
pH H_2O	7.7 ± 0.05			7.67 ± 0.02			6.18 ± 0.04			
pH KCl	7.52 ± 0.09			7.54 ± 0.01			6.15 ± 0.05			
%C	1.5 ± 0.1			2.6 ± 0.1			22.2 ± 0.6			
%N	0.07 ± 0.00			0.14 ± 0.0			1.6 ± 0.01			
CEC ($cmol_c$ kg^{-1})	31.31 ± 1.8			17.6 ± 1.4			57.6 ± 4.5			
P olsen (mg kg^{-1})	6.6 ± 0.5			46.1 ± 3.7			425.3 ± 33.0			
Metal concentrations (mg kg^{-1})	**Cd**	**Zn**	**Pb**	**Cd**	**Zn**	**Pb**	**Cd**	**Cu**	**Pb**	**Zn**
Pseudo-total	6.4 ± 0.6	3398 ± 221	234 ± 9	4.9 ± 0.3	2806 ± 132	292 ± 35	1.7 ± 0.1	301.9 ± 6.3	69.8 ± 2.6	433.5 ± 11.7
NH_4Cl extractable	4.43 ± 0.15	316.6 ± 5.9	2.22 ± 0.21	2.18 ± 0.18	122.0 ± 9.6	< loq	< loq	0.7 ± 0.0	< loq	1.5 ± 0.2

Loq: limit of quantification 2 mg kg^{-1} NH_4Cl-extractable Pb and 1 mg kg^{-1} NH_4Cl-extractable Cd.

Plant growth and metal accumulation in untreated and compost-amended soils

Plant biomass, shoot metal concentrations, metal yield and BCF for *N. tabacum* and *S. caprea* after the 3 month growth period in the untreated and compost-amended soils are summarised in Table 6.3. Shoot biomass of *S. caprea* in the untreated soil was 5-fold higher than that of *N. tabacum* ($p < 0.05$)). In the same soil, leaf Zn concentrations of *S. caprea* were almost 5-fold higher than stem concentrations ($p < 0.05$), and also significantly higher than shoot concentrations observed in tobacco ($p < 0.05$). In contrast, the shoot Cd concentrations of tobacco were significantly higher ($p < 0.05$) than either leaf or stem Cd concentrations in willow. Pb concentration in shoot tissues of *S. caprea* was under the quantification limit (1 mg kg^{-1}), while the mean shoot Pb concentration in *N. tabacum* was 6.7 mg kg^{-1}. The highest BCF for Zn corresponded with the leaves of *S. caprea* (6.7), while the highest BCF for Pb (6.4) and Cd (4.2) corresponded with *N. tabacum*. Both plant species grew better in the compost-amended soil compared to the untreated soil, but this was only significant for tobacco: compost amendment caused an increase of up to 85% in the biomass of tobacco ($p < 0.05$). At the same time, compost addition caused a significant decrease in metal (Zn, Cd and Pb) concentrations in tobacco shoots (between 2 to 3-fold decrease compared to plants grown in untreated soil; $p < 0.05$) and tended to reduce Zn and Cd concentrations in willow stems (1.2- and 1.5-fold decrease; $p > 0.05$). On the contrary, compost amendment significantly increased leaf Zn concentrations of *S. caprea* (from 2050 to 3840 mg Zn kg^{-1}; $p < 0.05$) and slightly increased the leaf Cd concentration (from 7.5 to 8.4 mg kg^{-1}). BCF values of *N. tabacum* were not strongly influenced by the addition of compost, while the BCF of *S. caprea* leaves increased for both Zn and Cd (by up to 3.6- and 1.4-fold, respectively).

The compost-induced increase in the growth of *N. tabacum* resulted in a 2-fold increase in Zn removal and a 3-fold increase in Cd and Pb removal compared to metal removal in the untreated soils. This effect was statistically significant for all three elements ($p < 0.05$). *Salix caprea* growing in the untreated soils were able to extract significantly more Zn and Cd than *N. tabacum* (around 3 -fold more; $p < 0.05$) due to the higher shoot biomass production. In the case of *S. caprea*, compost amendment also caused a significant increase in phytoextracted Zn ($p < 0.05$). However, contrary to what was observed for tobacco, in this case the increase in Zn removal was not related to an increase in biomass production but to an increase in shoot metal concentration. On the other hand, phytoextracted Cd by willows was significantly reduced after compost addition ($p < 0.05$).

Table 6.3. Comparison of the two plant species (*Nicotiana tabacum* and *Salix caprea*) growing in the two soil treatments (untreated and compost-amended soils) without inoculation, after 3 month growth period. Asterisks indicate significant differences between soil treatments ($p < 0.05$) and for each soil treatment different letters indicate significant differences between plant species and/or plant tissues ($p < 0.05$)

		Nicotiana tabacum			*Salix caprea*			
Shoot biomass (g)	Untreated	1.7 ± 0.1 b			8.4 ± 1.7 a			
	Compost-amended	12.0 ± 0.6 * a			9.1 ± 0.6 a			
		Shoot			**Leaves**		**Stem**	
		Zn	Cd	Pb	Zn	Cd	Zn	Cd
[metal] mg kg^{-1}	Untreated	724 ± 70 b	12.2 ± 0.87 a	6.67 ± 0.55	2046 ± 269 a	7.5 ± 1.4 b	423 ± 54 b	5.6 ± 0.9 b
	Compost-amended	213 ± 9 * c	5.8 ± 0.1 * cb	2.64 ± 0.38 *	3839 ± 325 * a	8.4 ± 1.9 ab	360 ± 17 b	3.8 ± 0.3 c
BCF	Untreated	3.6	4.2	6.4	6.7	1.7	1.4	1.3
	Compost-amended	2.4	3.6	> 2.6	24.5	2.4	2.3	1.1
		Shoot			**Shoot**			
		Zn	Cd	Pb	Zn		Cd	
Metal yield (µg plant $^{-1}$)	Untreated	1240 ± 101 b	21.0 ± 1.2 b	11.6 ± 1.3	4119 ± 457 a		61.4 ± 12.4 a	
	Compost-amended	2559 ± 191 * b	69.4 ± 3.0 * a	31.7 ± 5.3 *	8510 ± 1217 * a		47.3 ± 6.9 b	

Effects of rhizobacterial strains on biomass production, plant physiological status and shoot ionome

Figure 6.1 shows the effects of the five selected bacterial strains on the shoot yield of *N. tabacum* and *S. caprea*. For tobacco, inoculation had a more pronounced effect on plants grown in compost-amended soils than in untreated soils.

In untreated soils, only strains P30 and P75 caused a slight increase in the shoot biomass. In contrast, in the compost-amended soils, all the strains increased shoot biomass; and this increase was significant for strains P87, P29, P30 and P75 (Figure 6.1; $p < 0.05$). Bacterial-induced promotion in biomass was less pronounced in *S. caprea*, and only strain P30 caused a slight increase in biomass but this did not reach statistical significance.

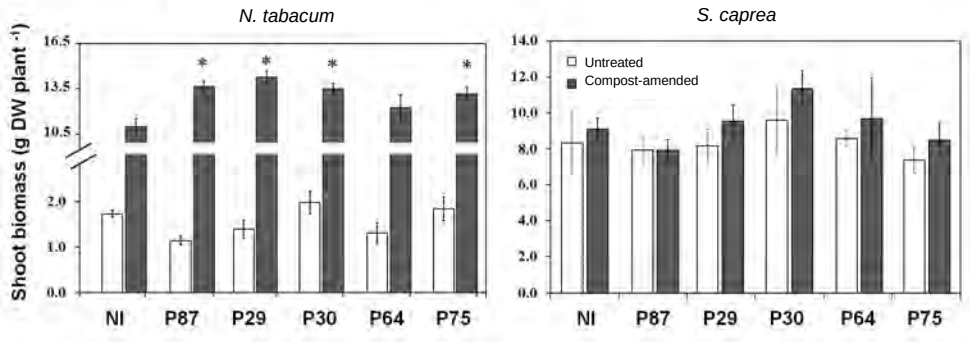


Figure 6.1. Effect of the five selected bacterial strains (P87, P29, P30, P64 and P75) compared to non-inoculated plants (NI) on the shoot yield (g) of *N. tabacum* and *S. caprea* grown in an untreated soil and compost amended soil. Asterisks indicate significant differences from the NI ($p < 0.05$).

The photochemical efficiency of plants was evaluated through the F_v/F_m, CHL and PRI (Figure 6.2). In general, in the untreated soil, *N. tabacum* leaves presented slightly higher values of F_v/F_m (0.86), CHL (1.42) and PRI (0.13) than *S. caprea* (0.75, 1.35 and 0.12, respectively). Compost addition had a beneficial effect on plant photochemical efficiency (reflected in F_v/F_m, CHL and PRI indices), and this was significant for all parameters in the case of willows and for the PRI index in the case of tobacco ($p < 0.05$). Significant effects of bacterial inoculation were found in the chlorophyll content after inoculating tobacco with strains P29, P30 and P75 and in the untreated soil (the CHL index increased from 1.42 in NI plants to 1.53, 1.53 and 1.52 in inoculated plants, respectively; $p < 0.05$). Moreover, strain P29 significantly increased F_v/F_m index in these plants (from 0.86 to 0.91; $p < 0.05$).

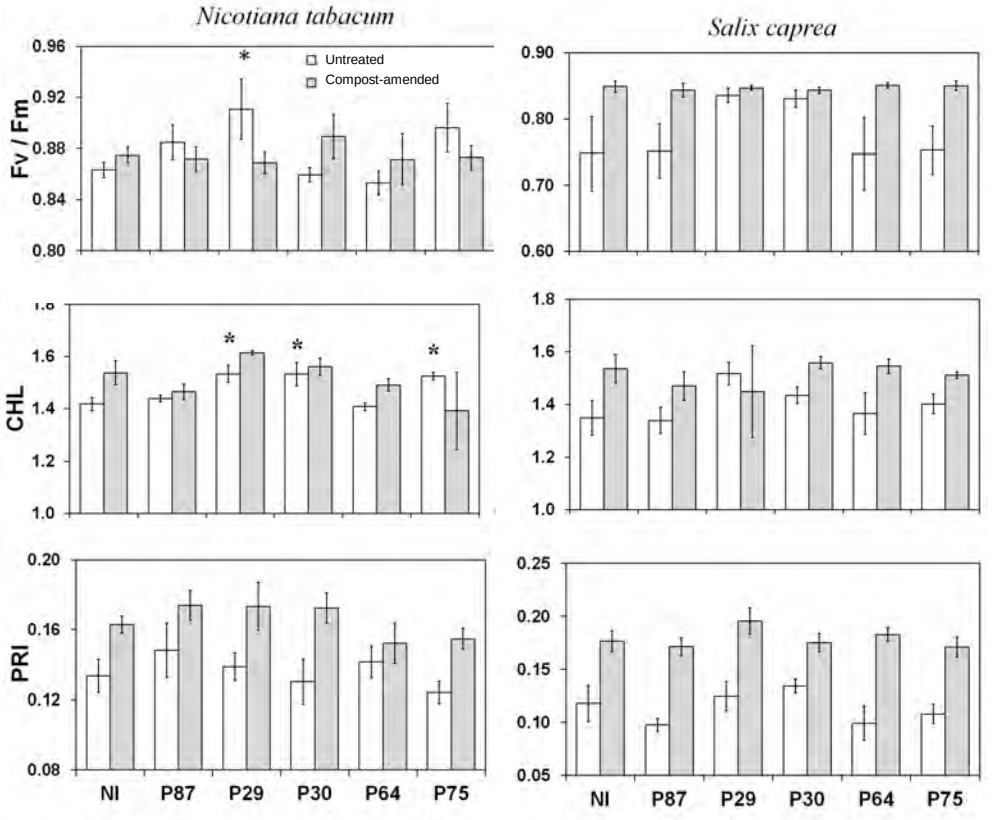


Figure 6.2 Effect of the five selected bacterial strains compared to NI plants on the mean values (mean ± SE) of chlorophyll content, as expressed by chlorophyll content index (CHL); PSII maximum quantum yield (Fv / Fm) and photochemical reflectance index (PRI) in *N. tabacum* and *S. caprea* grown in a untreated soil and compost amended soil. Asterisks indicate significant differences from the NI ($p < 0.05$)

Bacterial inoculation had some effect on shoot ionome particularly in plants growing in non-amended soils (Table 6.4), although in many cases these changes were not statistically significant and in general were not related to the strain used. Inoculation decreased the Mg and Ca concentration in tobacco when grown both in untreated or compost-amended soils (generally significantly for Ca; $p < 0.05$). A similar tendency (but not significant) was also detected in *S. caprea* leaves of plants growing in compost-amended soils. However, in for *S. caprea* growing in untreated soils, most strains tended to increase Ca and Mg concentrations in leaves and stems ($p > 0.05$). The bacterial treatments also tended to reduce the Fe content in tobacco grown in untreated soil, whereas an increase was observed when grown in compost-amended soil. In contrast, inoculation had a positive effect on Fe content in *S. caprea* grown in untreated soil, particularly in leaves where increases

Table 6.4. Effects of the five rhizobacterial strains in nutrient uptake by *N. tabacum* shoot and *S. caprea* shoot (leaves and stem). Asterisks indicate significant differences ($p < 0.05$)

Element (g kg^{-1})	Plant species		Soil treatment	Bacterial inoculant					
				NI	P87	P29	P30	P64	P75
K	*N. tabacum*		Unt	29.5 ± 1.1	36.0 ± 2.4	27.8 ± 1.3	21.8 ± 1.3 *	37.0 ± 2.5 *	25.8 ± 2.1
			Comp	26.5 ± 1.2	23.5 ± 0.5	23.8 ± 1.0	23.1 ± 0.5 *	27.7 ± 2.0	24.7 ± 0.8
	S. caprea	Leaves	Unt	9.6 ± 1.3	11.6 ± 2.6	7.3 ± 0.9	7.0 ± 1.1	12.4 ± 2.6	7.2 ± 0.9
			Comp	21.6 ± 3.1	21.3 ± 1.7	21.6 ± 4.6	20.3 ± 3.4	19.8 ± 4.0	22.0 ± 4.9
		Stem	Unt	3.5 ± 0.2	4.3 ± 0.6	4.3 ± 0.2	3.9 ± 0.3	3.6 ± 0.2	4.3 ± 0.2
			Comp	3.9 ± 0.2	4.7 ± 0.6	3.9 ± 0.2	3.7 ± 0.3	3.8 ± 0.3	4.4 ± 0.3
Ca	*N. tabacum*		Unt	33.2 ± 1.2	32.0 ± 1.8	29.5 ± 1.0	24.4 ± 2.0 *	29.8 ± 0.9	30.6 ± 0.9
			Comp	22.9 ± 0.4	20.1 ± 0.6 *	18.7 ± 0.4 *	17.7 ± 0.7 *	24.9 ± 1.4	20.1 ± 1.7
	S. caprea	Leaves	Unt	32.9 ± 4.7	37.3 ± 1.2	34.1 ± 1.3	28.1 ± 2.6	33.5 ± 3.4	33.4 ± 2.3
			Comp	47.6 ± 1.6	42.4 ± 1.6	42.8 ± 0.9	43.5 ± 2.5	44.8 ± 1.2	47.0 ± 1.7
		Stem	Unt	6.8 ± 0.8	7.8 ± 0.2	7.4 ± 0.7	7.1 ± 0.4	6.0 ± 0.3	7.7 ± 0.5
			Comp	5.4 ± 0.2	5.6 ± 0.3	6.0 ± 0.4	4.8 ± 0.17 *	5.4 ± 0.4	6.8 ± 0.8
Mg	*N. tabacum*		Unt	5.41 ± 0.33	5.07 ± 0.12	4.56 ± 0.14	4.62 ± 0.27	4.99 ± 0.35	4.87 ± 0.15
			Comp	5.35 ± 0.23	4.46 ± 0.22 *	5.09 ± 0.14	5.10 ± 0.14	5.04 ± 0.21	5.12 ± 0.17
	S. caprea	Leaves	Unt	6.39 ± 0.97	6.43 ± 0.54	6.63 ± 0.24	6.42 ± 0.72	5.97 ± 0.76	6.85 ± 0.62
			Comp	6.90 ± 0.47	6.21 ± 0.58	6.73 ± 0.52	6.04 ± 0.59	6.40 ± 0.42	6.45 ± 0.72
		Stem	Unt	0.80 ± 0.06	0.95 ± 0.05	0.93 ± 0.09	0.88 ± 0.05	0.78 ± 0.04	0.97 ± 0.05
			Comp	0.93 ± 0.02	0.91 ± 0.11	0.93 ± 0.05	0.69 ± 0.04	0.83 ± 0.07	1.04 ± 0.11

(continued)

Element (g kg^{-1})	Plant species		Soil treatment	Bacterial inoculant					
				NI	P87	P29	P30	P64	P75
Fe	*N. tabacum*		Unt	0.59 ± 0.08	0.42 ± 0.06	0.49 ± 0.06	0.43 ± 0.02	0.42 ± 0.05	0.41 ± 0.03 *
			Comp	0.27 ± 0.03	0.37 ± 0.04	0.40 ± 0.09	0.30 ± 0.05	0.35 ± 0.04	0.43 ± 0.07
	S. caprea	Leaves	Unt	0.21 ± 0.03	0.57 ± 0.15 *	0.55 ± 0.09 *	0.39 ± 0.05 *	0.38 ± 0.02 *	0.33 ± 0.04 *
			Comp	0.18 ± 0.02	0.12 ± 0.01	0.17 ± 0.03	0.19 ± 0.04	0.11 ± 0.01 *	0.13 ± 0.02
		Stem	Unt	0.06 ± 0.01	0.15 ± 0.07 *	0.08 ± 0.01	0.09 ± 0.02	0.08 ± 0.01	0.23 ± 0.07 *
			Comp	0.06 ± 0.00	0.07 ± 0.01	0.12 ± 0.02 *	0.07 ± 0.00	0.05 ± 0.01	0.09 ± 0.01
P	*N. tabacum*		Unt	1.96 ± 0.03	1.76 ± 0.09	1.30 ± 0.09 *	1.46 ± 0.07 *	1.76 ± 0.16	1.51 ± 0.05 *
			Comp	2.40 ± 0.13	2.11 ± 0.04	2.26 ± 0.10	2.36 ± 0.12	2.44 ± 0.13	2.51 ± 0.09
	S. caprea	Leaves	Unt	1.05 ± 0.17	0.97 ± 0.20	1.17 ± 0.07	0.75 ± 0.10	1.83 ± 0.68	0.77 ± 0.12
			Comp	1.17 ± 0.23	0.84 ± 0.09	0.94 ± 0.11	0.84 ± 0.08	0.72 ± 0.13	0.83 ± 0.06
		Stem	Unt	1.01 ± 0.07	1.20 ± 0.07	1.24 ± 0.08	1.12 ± 0.07	1.10 ± 0.02	1.26 ± 0.06 *
			Comp	1.54 ± 0.05	1.47 ± 0.13	1.49 ± 0.11	1.16 ± 0.05	1.42 ± 0.13	1.68 ± 0.18

Unt: Untreated soil; Comp: Compost-amended soil

of up to 2.7-fold with strain P87 were observed ($p < 0.05$). Bioaugmentation decreased P content in tobacco grown in untreated soil, particularly with strains P29, P30 and P75 ($p < 0.05$). The same tendency was observed in leaves of *S. caprea* grown in compost-amended soil and, albeit less markedly, also in untreated soils. Curiously the opposite effect was found in the stems of *S. caprea* grown in untreated soil, where in the case of P75 a significant increase in stem P concentration was observed ($p < 0.05$). Strains P64 and P87 induced an increase in K content of tobacco growing in untreated soils (significant for P64, $p < 0.05$). On the contrary, strain P30 significantly reduced K content in tobacco grown in both untreated and compost-amended soils. No clear effects of inoculants on K concentrations were observed for *S. caprea*.

Effects of bacterial inoculants on soil metal availability and fractionation

Metal soil availability was assessed at the end of the experiment after extraction with NH_4NO_3. Some bacterial-induced changes in metal availability were observed, and these were more pronounced in the untreated soil. After growth of *N. tabacum* all the bacterial strains significantly increased available Zn and Cd in this soil ($p < 0.05$) (Figure 6.3). Strain P75 showed the highest ability to increase Zn availability (NH_4NO_3-extractable concentrations increased from 30.7 mg kg^{-1} in NI soil to 39.8 mg kg^{-1} after inoculation), while strain P29 caused the highest increase in Cd availability (increasing from 0.16 mg kg^{-1} to 0.26 mg kg^{-1}). No bacterial-induced changes were detected for Zn in the compost-amended soils, while for Cd, strains P87, P30 and P75 significantly decreased the availability of this element ($p < 0.05$). In the case of *Salix*, strain P87 significantly increased soil Zn availability (from 32.1 mg kg^{-1} to 36.9 mg kg^{-1} ($p < 0.05$)), and all strains (except P87) significantly increased Cd availability in the untreated soils ($p < 0.05$) (Figure 6.3). The most pronounced increase was found for P29: NH_4NO_3-extractable concentrations increased from 0.16 mg Cd kg^{-1} in NI soil to 0.24 mg Cd kg^{-1} after inoculation. In the compost-amended soils strains P64 and P29 significantly increased soil Zn and Cd availability, respectively (from 10.1 mg kg^{-1} to 13.8 mg kg^{-1} and from 0.14 mg kg^{-1} to 0.18 mg kg^{-1} in the case of Zn and Cd, respectively).

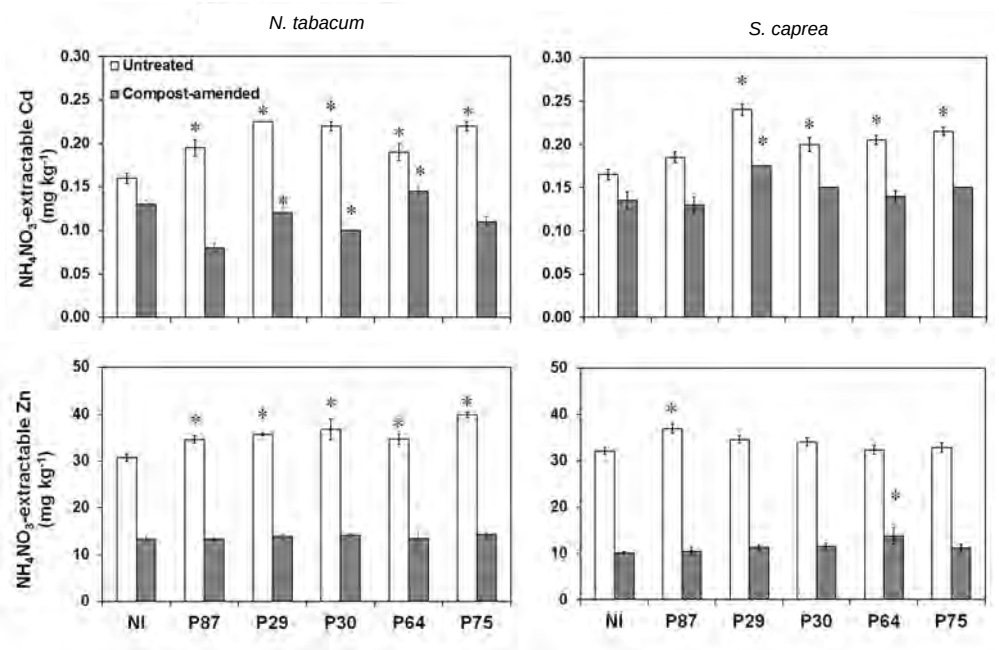


Figure 6.3. Effects of bacterial inoculants on metal availability (NH_4NO_3 –extractable metals) in *N. tabacum* and *S. caprea* planted soils. Asterisks indicate significant differences from the NI plants ($p < 0.05$)

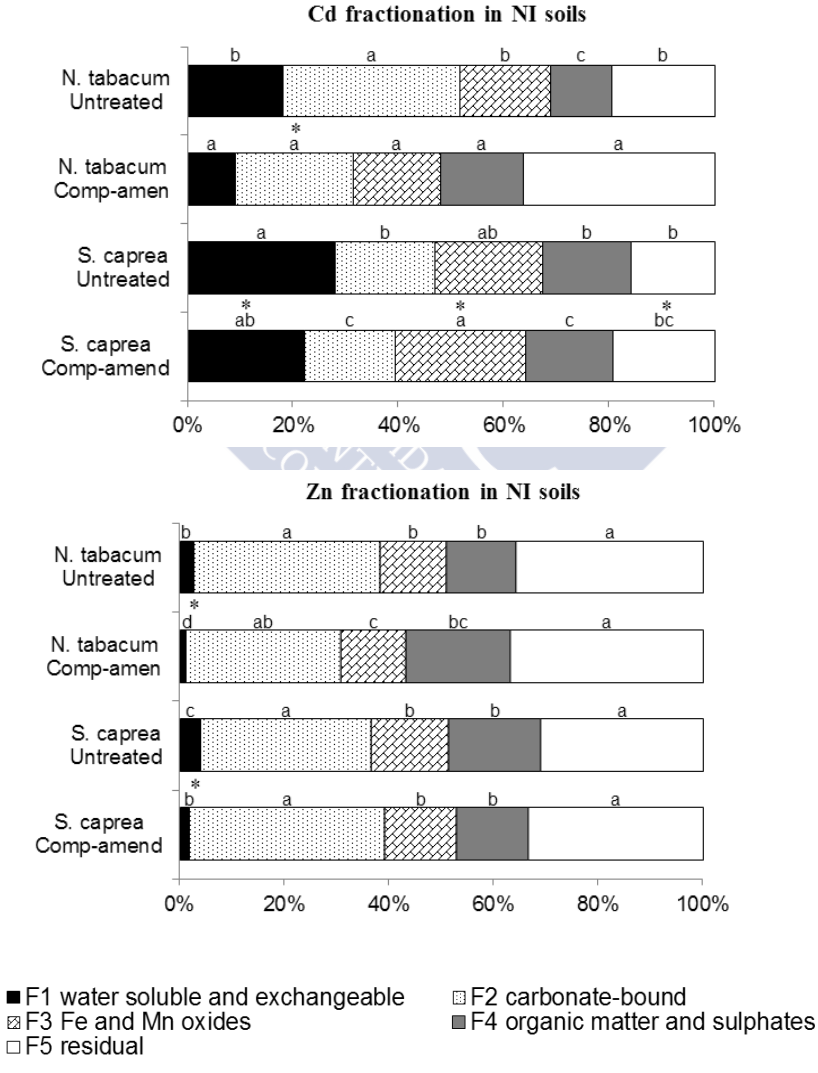
Figure 6.4 shows the soil metal fractionation (each fraction is represented as the % of the total) after plant growth in the NI treatment (Figure 6.4a), as well as the bacterial-induced depletion / increase for each fraction relative to the NI treatment (Figure 6.4b). Only those cases where a bacterial-induced effect on metal fractionation was observed are presented.

In the untreated soils and after plant growth, Zn was mainly present in the acid-extractable fraction (2600 mg kg^{-1} and 2480 mg kg^{-1} in *N. tabacum* and *S. caprea* planted soils, respectively) and residual phases (2590 mg kg^{-1} and 2360 mg kg^{-1} in *N. tabacum* and *S. caprea* planted soils, respectively). In the compost-amended soils a reduction of about 50% was observed in the most labile Zn fraction (F1) and an increase in the residual fraction. In these soils the F2 fraction increased and F4 decreased after the growth of *S. caprea*; conversely the F2 fraction decreased and F4 increased after growth of *N. tabacum*. Nevertheless, the dominant Zn pools remained the same. In untreated soils after the growth of either plant species about 50% of the total Cd was soluble and exchangeable (2.9 mg kg^{-1} and 4.4 mg kg^{-1} in *N. tabacum* and *S. caprea*, respectively) or acid-extractable (5.4 mg kg^{-1} and 3.2 mg kg^{-1} in *N. tabacum* and *S. caprea*, respectively). While in compost-amended soils these two fractions decreased and the reducible and

residual Cd fraction increased. Moreover, a 1.7-fold increase in the oxidisable Cd fraction (F4) was also detected in the case of tobacco.

Bacterial inoculation significantly influenced soil metal fractionation, and these changes depended on both the bacterial strain and the soil treatment. In untreated soils where *N. tabacum* was grown, strains P29 and P30 significantly increased the Zn concentration in the most plant-available F1 fraction (from 191 mg kg^{-1} in NI soils to 232 mg kg^{-1} and to 218 mg kg^{-1}, respectively).

(a)



(b)

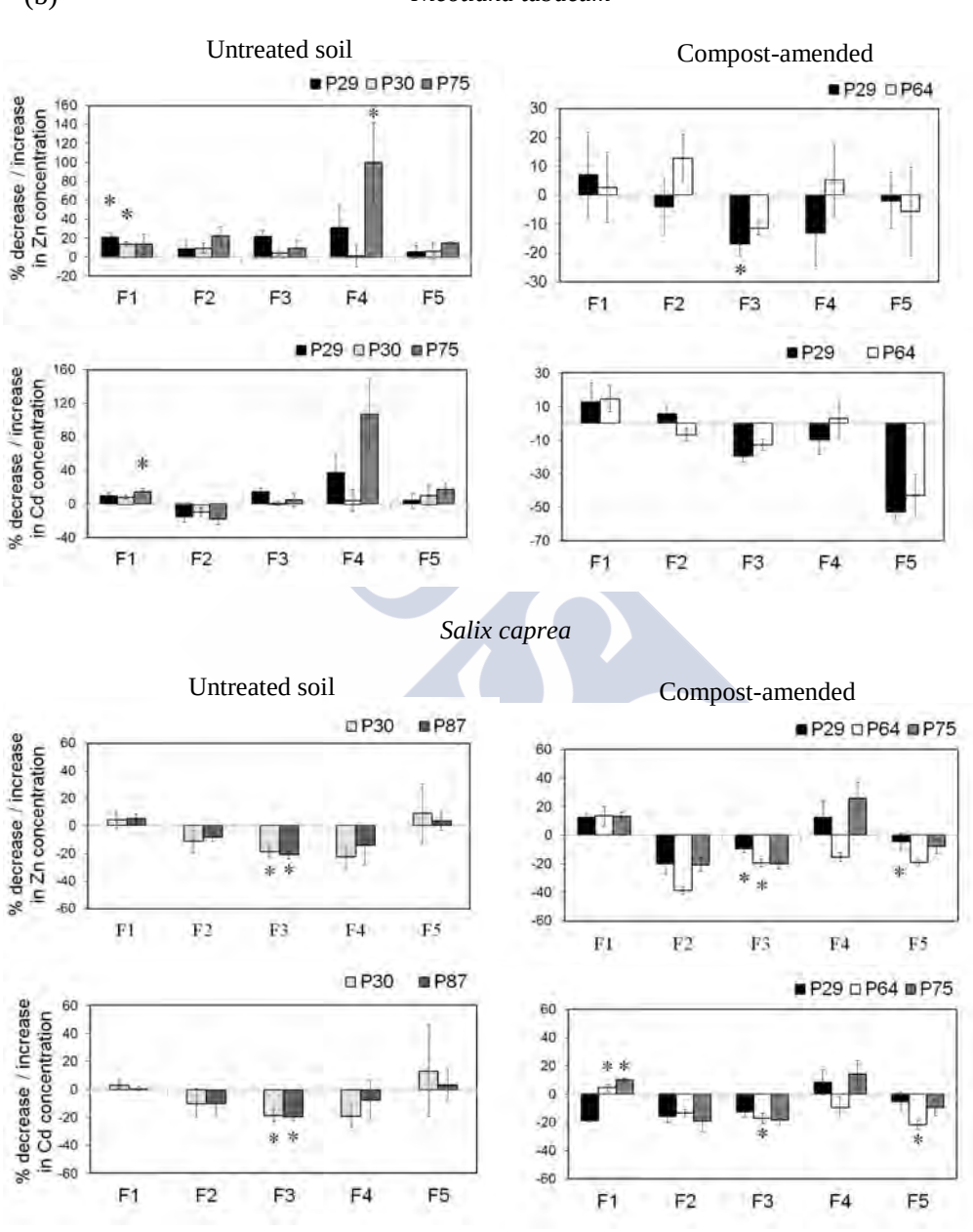


Figure 6.4. Metal fractionation in the NI soil (a) and mean relative (percent) bacterially induced depletion / increase of each fraction relative to the NI treatment (n = 6) in *N. tabacum* and *S. caprea* planted soils (b). Different letters in metal fractionation indicate significant differences between different fractions and asterisks indicate a significant effect of the compost on each fraction and plant species ($p < 0.05$). The depletion / increase was calculated by subtraction of the amounts of metals in each fraction at bacterial treatment from the amount in the non-inoculated treatment. A significant depletion / increase in the metal concentration is denoted with an asterisk ($p < 0.05$)

Similarly, strain P75 significantly increased the concentration of Cd in the F1 fraction (from 2.9 mg kg^{-1} to 3.4 mg kg^{-1}) (Figure 6.4b). In the same soil, strain P75 significantly increased the oxidisable (F4) fraction of Zn by up to 100%. The acid-extractable (F2) Cd was the only fraction where a bacterial-induced reduction was found after inoculation with all the strains (although this effect was not significant).

In untreated soils where *S. caprea* was grown, similar effects of strains P87 and P30 were found on soil Zn and Cd fractionation (Figure 6.4b). Both strains reduced the F2-F4 fractions, and this decrease was significant for both metals in the case of F3 (reducible phase). These metals were redistributed amongst the soluble (F1) and residual phases (F5). In the compost-amended soils, bacterial effects were less pronounced. In general, the bacterial inocula increased water soluble and exchangeable metal pools and decreased the acid-extractable, reducible and residual fractions (this was more pronounced for *S. caprea* where many of these alterations were statistically significant).

Effects of rhizobacterial inoculants on shoot metal concentrations and metal yield of Nicotiana tabacum and Salix caprea

Shoot metal concentrations of harvested tobacco are presented in Figure 6.5. In untreated soils, strains P29, P30 and P75 decreased the shoot metal concentration of the three major contaminating metals (Zn, Cd and Pb), and this effect was significant for Pb after inoculation with strains P30 and P75 ($p < 0.05$)). The effects of strains P87 and P64 were metal-dependent but not significant. For example, these inoculants increased shoot Zn concentrations, but they did not influence the shoot Cd concentration and slightly decreased shoot Pb concentration. In the compost-amended soils, bacterial inoculation did not significantly affect shoot Zn concentration, while strains P29 and P30 strains significantly decreased the Cd concentration ($p < 0.05$). Metal concentrations in *S. caprea* were determined for leaves and stems (Figure 6.6). Higher metal concentrations, and more pronounced bacterial-induced effects, were found in the leaves compared to the stems. Strain P87 significantly increased leaf Cd concentration (from 7.5 mg kg^{-1} to 13.1 mg kg^{-1}; $p < 0.05$), in plants grown in untreated soils. On the other hand, as observed with tobacco plants, strains P29 and P30 decreased both these elements in the leaves of *S. caprea*. In the compost-amended soil none of the bacterial strains led to an increase in leaf metal concentrations. Leaf Zn and Cd concentrations of NI plants were up to 5- and 1.3-fold higher than stem

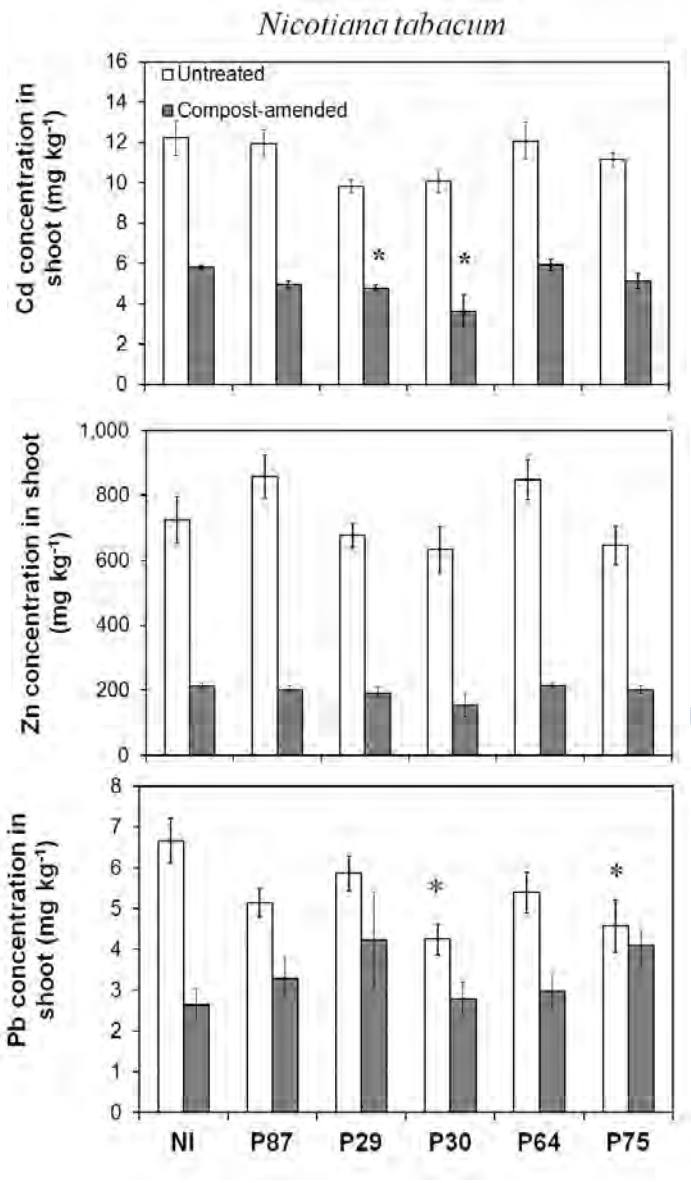


Figure 6.5. Effects of bacterial inoculants on shoot metal concentration of *N. tabacum* growing in untreated and compost-amended soils. Asterisks indicate significant differences from the NI plants ($p < 0.05$). White bars show shoot Zn, Cd and Pb concentrations in the untreated soils and grey bars indicate metal concentrations in plants growing in the compost-amended soils.

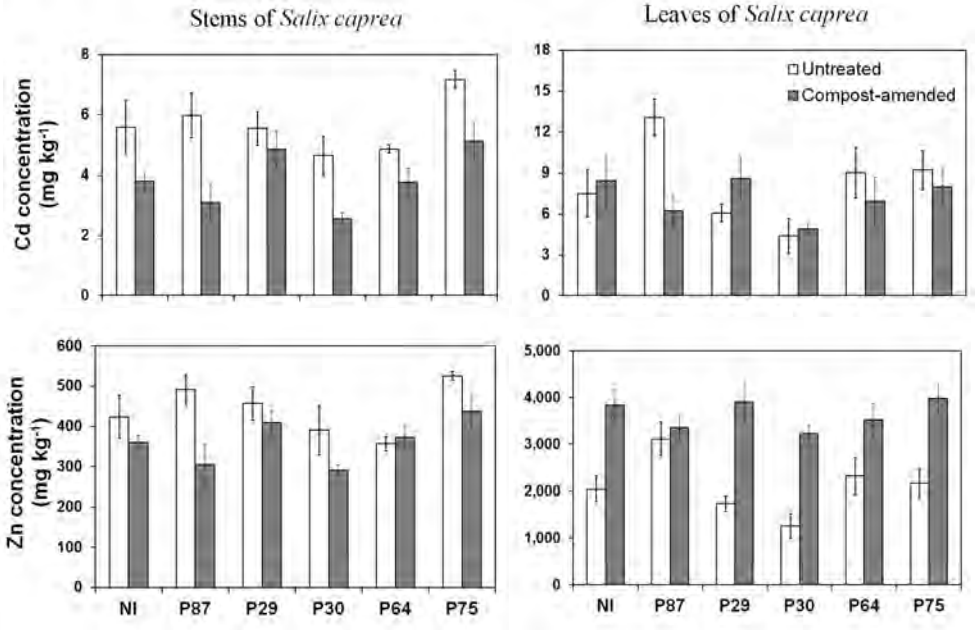


Figure 6.6. Effects of bacterial inoculants on leaves and stem metal concentration of *S. caprea* growing in untreated and compost-amended soils. Asterisks indicate significant differences from the NI plants ($p < 0.05$)

concentrations in untreated soils, and up to 10- and 2-fold higher in compost-amended soils, respectively. In untreated soils, strains P87 and P75 increased the stem Zn concentration, and only one strain (P75) led to an increase in Cd concentration. In contrast to what was observed in leaves, strains P29 and P75 led to an increase in the stem Cd and Zn concentration in willows growing in compost-amended soil ($p < 0.05$).

Effects of bacterial inoculation on *N. tabacum* metal yields were strongly influenced by the soil treatment (Table 6.5). In untreated soil, metal yield was negatively affected by bacterial inoculation and even a significant reduction was found for Pb after inoculation with P87 and P64 compared to non-inoculated plants (by up to 50% and 42%, respectively ($p < 0.05$)). However, in plants growing in the compost-amended soils, all five inoculants improved metal yields and this was the case for all three metals (although not significant). Strain P87 had the highest impact on Zn yields (increasing from 2559 μg plant^{-1} to 2994 μg plant^{-1} after inoculation), P64 on Cd yield (increasing from 69.4 μg plant^{-1} to 77.8 μg plant^{-1}) and P75 on Pb yield (increasing from 31.7 μg plant^{-1} to 58.9 μg plant^{-1}). For *S. caprea*, the beneficial effects of inoculation on metal yields were more pronounced in the untreated soil: strains P87, P29 and P75 induced a slight

Table 6.5. Effect of the five selected bacterial strains (P87, P29, P30, P64 and P75) compared to non-inoculated plants (NI) on the metal yield (µg) of *N. tabacum* and *S. caprea* grown in an untreated soil and compost amended soil. Asterisks indicate significant differences from the NI ($p < 0.05$)

	N. tabacum Yield (µg)			*S. caprea* Yield (µg)	
	Cd	**Zn**	**Pb**	**Cd**	**Zn**
Non-amended soil					
NI	21.0 ± *1.2*	1241 ± *102*	11.6 ± *1.3*	61.4 ± *12.4*	4120 ± *451*
P87	13.5 ± *0.9*	961 ± *57*	5.9 ± *0.7* *	56.1 ± *4.2*	4429 ± *267*
P29	13.6 ± *1.9*	925 ± *120*	8.4 ± *1.7*	57.6 ± *6.0*	4429 ± *138*
P30	20.1 ± *2.8*	1217 ± *142*	8.3 ± *1.0*	65.2 ± *12.4*	3471 ± *286*
P64	15.3 ± *2.0*	1068 ± *125*	6.7 ± *0.8* *	61.5 ± *2.4*	3635 ± *360*
P75	20.3 ± *2.6*	1133 ± *92*	8.0 ± *1.1*	52.1 ± *5.2*	4241 ± *312*
Compost-amended soil					
NI	69.4 ± *3.0*	2560 ± *192*	31.7 ± *5.3*	47.3 ± *6.9*	8511 ± *1217*
P87	74.1 ± *3.5*	2994 ± *246*	49.3 ± *8.4*	28.6 ± *6.2*	4454 ± *945*
P29	74.2 ± *2.0*	2960 ± *209*	49.3 ± *10.2*	54.1 ± *3.4*	8121 ± *593*
P30	66.7 ± *3.9*	2750 ± *128*	41.2 ± *5.7*	31.0 ± *2.0*	4951 ± *660*
P64	77.8 ± *2.8*	2840 ± *107*	39.8 ± *6.9*	42.2 ± *7.8*	6665 ± *1528*
P75	73.9 ± *6.5*	2901 ± *226*	58.9 ± *8.4*	49.9 ± *8.5*	6953 ± *1357*

increase in the Zn yield compared to NI plants (from 4119 mg plant^{-1} in NI plants to 4429, 4429 and 4241 mg plant^{-1}, respectively; $p > 0,05$), while strain P30 increased the Cd yield (from 61.4 µg plant^{-1} to 65.2 µg plant^{-1}; $p > 0,05$). However, in compost-amended soils bacterial inoculants generally reduced the Zn and Cd yield, with the exception of strains P29 and P75.

6.4. Discussion

This study evaluated the benefits of two strategies, compost amendment and/or bioaugmentation, on the metal phytoextraction efficiency of *N. tabacum* and *S. caprea*. Tobacco grew poorly in the untreated soil, and this was most likely related to nutrient deficiency and metal phytotoxicity. The addition of compost had a strong promoting effect on tobacco growth, probably due to improvements in mine-soil fertility and structure and possibly a reduction in metal phytotoxicity. The increase in some of the evaluated indices related to the plant physiological

status confirmed this reduction in plant stress. A positive response in growth and biomass production of the same tobacco clone was also observed after the application of inorganic (mainly N) fertilisers in smelter- and sewage sludge- affected TE-contaminated agricultural soils (PHYTAC, 2005). Improvements in soil properties and promotion of plant biomass by compost addition are widely described (van Herwijnen *et al.,* 2007; Alvarenga *et al.,* 2009a; Alvarenga *et al.,* 2009b; Alburquerque *et al.,* 2011; Parraga-Aguado *et al.,* 2015). The incorporation of these organic wastes into phytomanagement processes can contribute towards the recycling of residues, thus supporting current European policies aimed at reducing wastes (EU's Zero Waste Policy, 2013) and may overcome the need for inorganic fertilisation. Our results suggest that, provided soil amendments (such as compost) are incorporated, tobacco could be cultivated in contaminated mine-soils. However, field and economic assessments would be required to determine its full potential for the phytomanagement of this type of site.

The aerial biomass of *S. caprea* was almost 5-fold higher than tobacco plants in the untreated soil, and the compost addition only led to a slight but not significant increase in biomass production (although plants grown in compost-amended soils generally showed higher photosynthetic efficiency). In contrast, French *et al.* (2006) found a large stimulation in the yield of several woody crops (including *Salix*) after amending a TE-contaminated landfill site with sewage. However, *Salix caprea* is well known to colonise soils with edaphically extreme properties, probably due to their low nutritional requirements (Dickinson *et al.*, 1994). Several authors have reported a better survival rate and growth of *S. caprea* in contaminated soils compared to other *Salix* species (Dos Santos Utmazian and Wenzel 2007; Dos Santos Utmazian *et al.*, 2007b). Our results confirm the metal tolerance of this species and its potential for re-vegetation of mine-soils.

Compost addition significantly decreased CEC and this reduction was possibly due to the occlusion of exchanges sites by added organic matter. Moreover, compost had a significant effect on soil metal availability and fractionation. Mineralisation of organic matter after its incorporation into the soil can frequently lead to a reduction in soil pH but this was not observed here. Alburquerque *et al.* (2011) also found no changes in the soil pH of neutral soils after compost addition. The effects of organic matter additions on metal availability and fractionation are generally described as being pH dependent. The formation of soluble organo-metal complexes both at high or low pH can increase

metal solubility (Gregson and Alloway, 1984); although according to Jahiruddin *et al.* (1985) this pH-induced solubilisation effect could be inhibited in calcareous soils. In this study, we found approximately a 2-fold decrease in the availability of Zn, Cd and Pb after organic amendment addition which could not be associated with a pH change. Other edaphic factors such as the nature of the organic matter, soil type or the element characteristics will also influence soil metal mobility (Clemente *et al.,* 2005; Kumpiene *et al.,* 2011). Although several authors have reported increases in soil metal availability after compost additions (related to the high dissolved organic matter (DOM) content in some types of unstabilised organic matter or to the high capacity of metal complexation by humic compounds (Clemente and Bernal, 2006; Tandy *et al.,* 2009)) it is more common to find an immobilising effect of organic amendments in the literature (Clemente *et al.,* 2005; van Herwijnen *et al.,* 2007; Alburquerque *et al.,* 2011). This reduction in metal mobility is often attributed to the stabilisation of organic wastes after the composting process which reduces the DOM content (Kiikkilä *et al.,* 2002) or to the high proportion of humified organic matter in the amendments, which can decrease the metal mobility through adsorption and formation of stable complexes with humic substances (Shuman, 1999). The presence of other compounds in the compost such as phosphates or Fe/Al (hydro)oxides could also lead to the precipitation of metals through the formation of insoluble metal complexes (e.g. Pb pyromorphites) (Brown *et al.*, 1998). Organic amendments can also lead to a re-distribution in trace metal fractionation from soluble and exchangeable forms to pools associated with OM, iron oxides or the residual fraction (Shuman, 1999; Clemente and Bernal, 2006; Alvarenga *et al.,* 2009a; Alburquerque *et al.,* 2011; Kumpiene *et al.,* 2011). Here, there was a shift of metals from the more available to less available fractions after addition of 5% (w/w) compost, and this was especially pronounced for Cd.

In previous studies, the tobacco clone NBCu 10-8 has been shown to present a high capacity for Cd and Zn accumulation: Herzig *et al.* (2014) found that this clone accumulated up to 293 mg Zn kg^{-1} in its aboveground biomass when growing in a mainly Zn-contaminated soil polluted with deposits of a former hot dip Zn factory at Bettwiesen in eastern Switzerland. The same tobacco clone accumulated up to 15 mg Pb kg^{-1} and 4 mg Cd kg^{-1} after adequate inorganic fertilisation (mainly N) at the Rafz site in Switzerland, and between 300 to 400 mg Zn kg^{-1} and 10 to 20 mg Cd kg^{-1} at the Balen site in Belgium (PHYTAC, 2005). These sites are agricultural soils contaminated by sewage sludge application (Rafz) or by

fall-out from a Zn/Pb smelter (Balen). The shoot Cd and Pb concentrations of the tobacco clone NBCu 10-8 obtained here were of a similar magnitude to those obtained in these previous studies, while shoot Zn concentrations were even higher (2 to 3-fold) in the untreated soil (and of a similar magnitude in the compost-amended mine-soil).

Metal accumulation by different *Salix* species and/or clones is highly variable. Unterbrunner *et al.* (2007) found concentrations of up to 166 mg Cd kg^{-1}, 438 mg Pb kg^{-1} or 3000 mg Zn kg^{-1} in leaves of adult trees of *S. caprea* growing in long-term Cd- and Zn-contaminated areas across Central Europe. These leaf Cd concentrations are two orders of magnitude higher than those found in this study, however, the shoot Zn concentrations were similar to our data. Differences between studies in the metal accumulation by this clone could be attributed to differences in plant age, growth conditions and shoot biomass (i.e. short-term pot study compared to adult trees growing in the field), soil properties and TE concentration and availability. Metal accumulation was strongly compartmentalised in *S. caprea* and primarily occurred in the leaves. Higher concentrations of Cd and Zn in the leaves of Salicaceae have commonly been reported (Pulford and Watson 2003; Janssen *et al.*, 2015; Kidd *et al.*, 2015). Consequently many authors suggest harvesting both leaves and twigs to increase metal removal. Vyslouzilova *et al.* (2003) found that up to 83% and 71% of the total phytoextracted Zn and Cd by willows were in the leaves. At the Lommel site (Belgium) between 81 to 121 g Cd ha^{-1} yr^{-1} could be removed by poplars when harvesting only twigs and up to 187 to 240 g Cd ha^{-1} yr^{-1} if leaves were also harvested (Vangronsveld *et al.*, 2009).

Compost addition significantly decreased shoot metal concentration of *N. tabacum*, which mirrored the reduction in soil metal availability but could not be attributed to a dilution effect since metal yield was not reduced. In contrast, despite the reduction in soil metal availability compost addition had no effect on either stem Cd or Zn concentrations in *S. caprea*, and surprisingly, led to an increase in the leaf Zn concentration of this species. Compost addition can clearly increase soil organic matter content and fertility thus improving plant growth in mine-soils; however, given their effects on metal availability it will be important to optimise the rates of addition if metal extraction is the targeted process. Long-term field experiments are needed to evaluate the duration of the effects of compost addition. Moreover, it would be interesting to compare the effectiveness of these amendments after one single high compost dressing before planting with

that of repeated additions of lower doses. Phytoextraction efficiency in terms of Zn yield was consistently higher for *S. caprea* than *N. tabacum*. Moreover, the addition of compost effectively increased Zn yield of *S. caprea* even further, due to the observed increase in leaf Zn concentration. However, compost addition significantly improved the Cd (and Zn) yields of *N. tabacum* and these reached values close to those of *S. caprea*. Moreover, it is important to consider that when applied in practice willow SRC are only harvested every 3-5 years, while tobacco is harvested annually, and therefore the phytoextraction efficiency of these two species may differ under field conditions.

The second strategy evaluated was the use of bioaugmentation to improve phytoextraction efficiency. The effect of bacterial strains on plant growth or metal accumulation is highly soil- and plant species-specific (Grandlic *et al.*, 2008; Becerra-Castro *et al.*, 2012; Cabello-Conejo *et al.*, 2014). Likewise, we found that bacterial-induced modifications in metal availability, plant accumulation and plant growth varied according to both the soil treatment (with or without compost) and the plant species (*S. caprea* or *N. tabacum*). Moreover, the effects of bacterial inoculants on metal accumulation differed in the leaf or stem tissues of *S. caprea*. Strain P30 (*Rhodococcus erythropolis*) showed a generalised positive effect on the growth of both plant species; while strains P75 (*Tsukamurella strandjordii*), P87 (*Massilia niastensis*) and P29 (*Pseudomonas costantinii*) were only found to enhance growth of *N. tabacum* and sometimes photochemical efficiency (CHL and F_V/F_m). Genera such as *Rhodococcus* or *Pseudomonas* have been frequently described as PGPR (Dell'Amico *et al.*, 2008; Becerra-Castro *et al.*, 2013; Langella *et al.*, 2014; Janssen *et al.*, 2015). The most pronounced stimulation in biomass was recorded for tobacco growing in the compost-amended soil. Grandlic *et al.* (2008) also found a more pronounced beneficial effect of bacterial inoculants on the growth of *Atriplex lentiformis* in compost-amended soil (with a compost addition of 10% w/w) compared to untreated mine-soil. These authors attributed this effect to the requirement of the bacterial strains for a minimal amount of organic matter for their successful establishment in the soil and protection against metal toxicity. No phenotypic traits were found to be consistently associated with those strains which promoted plant growth, although two strains (P30 and P75) produced IAA. Strain P29 (*Pseudomonas* sp.) which showed the highest growth promotion was able to solubilise inorganic phosphate and biosurfactants, but was unable to produce IAA. Furthermore, some strains increased plant photosynthetic parameters (chlorophyll content and photosynthetic efficiency) and these effects

were more pronounced in tobacco, although in this case the bacterial effects were not related to the presence of compost amendment in the soil. It would be interesting to carry out further studies evaluating the effects of these rhizobacterial inoculants and their ability to reduce plant stress in TE-contaminated mine-soils such as those used here.

Becerra-Castro *et al.* (2012) found all five strains tested in this study to increase the growth of *F. pratensis*, and the strains P29, P30 and P87 to increase the growth of *S. viminalis*, when grown in perlite with hydroponic culture supplemented with Cd and Zn. However, they found no bacterial-induced increase in metal accumulation by either plant species (Becerra-Castro *et al.*, 2012). Here, only tendencies regarding the effects of bacterial strains on plant metal accumulation were observed. In general, strain P87 increased shoot metal concentrations and strain P30 reduced metal accumulation by plants. For the rest of the bacterial strains the effects were soil-dependent. Bacterial-enhanced soil metal mobility (assessed through NH_4NO_3-extraction) was more pronounced in the case of untreated soils. A possible explanation could be that due to the inherent low fertility of the soil, the bacterial strains use their capacities to produce and release organic acids, siderophores, or P solubilisation to obtain nutrients and indirectly promote metal mobilisation. However, the increase in Cd or Zn extractable concentrations did not always correlate with an increase in shoot metal concentration or in metal yield. It is well known that plants experiment seasonal variations in metal uptake and accumulation and longer growth periods may be necessary to observe the consequences of an increased soil metal availability on shoot metal concentrations. Harvesting of the plants before reaching their maximum period of activity and metal uptake could explain the lack of correlation between the changes in soil metal availability and shoot metal accumulation.

Overall, the use of compost had a more beneficial effect on phytoextraction efficiency than the use of bacterial inoculants alone (i.e. inoculated untreated soil). Although the use of both, compost addition and bacterial inoculants in combination, tended to increase plant metal yields, some plant species-bacterial strain combinations did not lead to a significant increase in phytoextraction efficiency.

6.5. Conclusions

This study evaluated two strategies (addition of organic amendments and bacterial inoculation) to improve the overall process of phytoextraction. Both Cd and Zn phytoextraction efficiency were enhanced due to an increase in plant biomass production and/or plant metal concentration, but the benefits were plant-species specific. Compost amendment significantly increased shoot DW yield in the case of tobacco and leaf Zn concentration in *S. caprea*, while the bacterial strain P87 significantly increased leaf Cd accumulation in *S. caprea* growing in untreated soils. Moreover, in some cases the combined effects of both strategies were more beneficial than when used on their own. Bacterial inoculation markedly improved growth of *N. tabacum* in the compost-amended soils, leading to an overall higher metal removal. Few studies have evaluated the effects of bacterial inoculants in combination with soil amendments and using more than one host plant species. The identification of potential bacterial strains (such as P87) which are able to respond under a range of soil conditions is interesting for transferring these strategies to real-life field scenarios. Moreover, those bacterial strains which acted in synergy with the compost amendment to enhance shoot biomass could on a longer term lead to higher shoot metal removal. However, further studies are required to fully evaluate aspects such as the effect of the combination of various strains in a bacterial consortium, the optimal method of inoculation, the need for re-inoculation events, as well as the persistence and competition capacity of inoculant strains in the soil.

6.6. References

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Chapter 7

Inoculation methods using *Rhodococcus erythropolis* strain P30 affects bacterial assited phytoextraction capacity of *Nicotiana tabacum*

ABSTRACT

In this study different bacterial inoculation methods were tested for tobacco plants growing in a mine-soil contaminated with Pb, Zn and Cd. The inoculation methods evaluated were: seed inoculation, soil inoculation, dual soil inoculation event and seed+soil inoculation. Each inoculum was added at two bacterial densities (10^6 CFUs mL^{-1} and 10^8 CFUs mL^{-1}). The objectives were to evaluate whether or not the mode of inoculation or the number of applied microorganisms influences plant response. The most pronounced bacterial-induced effect was found for biomass production, and the soil inoculation treatment (using 10^6 CFUs mL^{-1}) led to the highest increase in shoot dry weight yield (up to 45%). Bacterial-induced effects on shoot metal concentrations were less pronounced; although a positive effect was found on shoot Pb concentration when using 10^8 CFUs mL^{-1} in the soil inoculation (29% increase) and in the seed+soil inoculation (34% increase). Also shoot Zn concentration increased by 24% after seed inoculation with 10^6 CFUs mL^{-1}. The best effects on the total metal yield were not correlated with an increasing number of inoculated bacteria. In fact the best results were found after a single soil inoculation using the lower cellular density of 10^6 CFUs mL^{-1}.

This study formed part of the following publication:

Álvarez-López, V., Prieto-Fernández, A., Janssen, J., Herzig, R., Vangronsveld, J., Kidd, P.S. (2016). Inoculation methods using *Rhodococcus erythropolis* strain P30 affects bacterial assisted phytoextraction capacity of *Nicotiana tabacum*. *Int J Phytorem* 18:406-415.

7.1. Introduction

Phytomanagement of trace metal-contaminated soils describes a series of plant-based remediation technologies which aim to reduce associated environmental risks and restore soil functions and quality (Mench *et al.*, 2009; Robinson *et al.*, 2009; Vangronsveld *et al.*, 2009). Phytoextraction is based on the use of plants that accumulate large amounts of metals into their above-ground biomass which can then be harvested. Metal removal depends on the metal concentration in harvestable plant parts and biomass production (Kidd *et al.*, 2009; Robinson *et al.*, 2009; Vangronsveld *et al.*, 2009). High-biomass crops and woody plants are considered good candidates for phytoextraction of trace metals (particularly Cd, Se and Zn) if they also show relevant shoot metal removals and a high shoot yield. Moreover, metal extraction can be combined with bioenergy production (Schroder *et al.*, 2008; Nsanganwimana *et al.*, 2014; Kidd *et al.*, 2015).

Phytoextraction efficiency can be limited by reduced plant biomass production or low soil metal bioavailability (Baker *et al.*, 2000; Wenzel, 2009). Several strategies have been proposed to optimise the process, such as using improved plant cultivars, appropriate agronomic techniques, or beneficial plant-associated microbial inoculants (Nehnevajova *et al.*, 2007; Vangronsveld *et al.*, 2009; Glick, 2010; Kidd *et al.*, 2015). Microbial-assisted phytoextraction uses native microorganisms which enhance plant growth and/or alter soil metal availability (Glick, 2010; Ma *et al.*, 2011; Janssen *et al.*, 2015). Numerous authors have isolated bacterial strains from metal-rich or metal-contaminated areas, screened these for their plant growth promoting (PGP) traits and metal resistance, and used them as plant inoculants (Haslmayr *et al.*, 2014). In many cases a bacterial-induced enhancement in plant biomass production and hence metal removal has been shown, and several strains have been reported to be useful for phytoextraction (see review by Sessitsch *et al.* (2013)). Phytoextraction efficiency is enhanced through a bacterial-induced increase in soil metal mobility (Sessitsch *et al.*, 2013) or by increasing plant growth (plant growth promoting bacteria (PGPB) (Glick, 2014; Visioli *et al.*, 2015). It is generally accepted that the experimental conditions, in particular the host plant species, soil type and properties, and nature and type of contamination (mono- or poly-metallic contamination; co-contamination with organic and inorganic pollutants; pollutant concentration; anthropogenic, geogenic or spiked contamination), are important factors determining whether or not a beneficial bacterial-induced effect is observed (Weyens *et al.*, 2010; Becerra-Castro *et*

al., 2012; Sessitsch *et al.*, 2013; Cabello-Conejo *et al.*, 2014). However, few studies have evaluated the effects of the inoculation method on plant growth and metal accumulation. Inoculation methods differ greatly between studies (inoculation of seed/plant/soil, number/timing of inoculation events, bacterial cellular densities) and these aspects are likely to influence the final effects of such plant-microbial associations. The most common methods used are seed and soil inoculation, while plant inoculation and experiments including more than one inoculation event are rare (Abou-Shanab *et al.*, 2006; Dell'Amico, Cavalca and Andreoni 2008; Becerra-Castro *et al.*, 2012; Cabello-Conejo *et al.*, 2014). Bacterial densities of the inoculum generally fluctuate between 10^7- 10^8 CFUs mL^{-1}, however the total density of inoculated bacteria per pot (CFUs per pot) is highly variable due to the different amounts of soil used (Kuffner *et al.*, 2010; Marques *et al.*, 2013; Petriccione *et al.*, 2013; Langella *et al.*, 2014). According to Malusá *et al*. (2012), the commercial use of microbial inoculants has been limited due to the inconsistency of the results obtained. There is therefore a need to test the effects of different inoculation methods on plant growth promotion and metal removal under standardised conditions.

The objectives of this study were to evaluate if the effects of a bacterial strain with PGP traits (*Rhodococcus erythropolis* P30) on metal phytoextraction capacity of *Nicotiana tabacum* are dependent on the inoculation method. For this, we assessed the effects on plant biomass production and metal accumulation of (i) seed versus soil inoculation, (ii) inocula with different bacterial densities: 10^6 versus 10^8 CFUs mL^{-1}, and (iii) the number of inoculation events (single versus repeated inoculation).

7.2. Materials and Methods

Soil and plant species selected for the pot experiment

Mine soil (< 20 cm depth) was collected from the tailings of the abandoned Pb-Zn mine of Rubiais in Lugo, NW Spain (UTM 29T 660781/4726800). The physicochemical characteristics of the tailings have been described in detail previously (Becerra-Castro *et al.*, 2012; Monterroso *et al.*, 2014). The mine soil was air-dried and sieved to < 8 mm and mixed with 5% compost (w/w). Compost addition of 5% (w/w) was necessary to achieve optimal growth of tobacco (Álvarez-López *et al.*, 2011). The compost was based on municipal sewage sludge and wood chippings. The compost presented a slightly acid pH (6.2), high C/N

ratio (13.5), high CEC (57.6 $cmol_c$ kg^{-1}), and a low to moderate concentration of trace metals. The mine-soil had a slightly alkaline pH (8.23), low cationic exchangeable capacity (18.0 $cmol_c$ kg^{-1}) dominated by Ca, low P availability (25.9 mg kg^{-1}) and high concentrations of total Cd, Zn and Pb (7, 5328 and 893 mg kg^{-1}, respectively). The NH_4Cl-extractable Cd and Zn concentrations were 1.84 and 201 mg kg^{-1}, respectively. The NH_4NO_3-extractable Cd and Zn concentrations were 0.23 and 33.7 mg kg^{-1}, respectively. Pb concentrations were below the quantification limit in both NH_4NO_3- and NH_4Cl-extractable fractions (0.15 and 2 mg kg^{-1}, respectively).

Tobacco can be used for metal phytoextraction and at the same time can provide biomass for bioenergy, green fine chemistry, and other ecotechnologies (Schroder *et al.*, 2008). In this study, the NBCu 10-8 variant of *Nicotiana tabacum* (F_2 generation) was used. This variant was selected from 106 somaclonal variants of tobacco by *in vitro* breeding techniques and showed an improved Cd and Zn extraction capacity compared to the mother line (Guadagnini, 2000; Herzig *et al.*, 2014). Plants were grown in 2.5 L pots (with 2 kg of soil).

Rhizobacterial strain and inoculation treatments

The strain P30 (*Rhodococcus erythropolis*; Accession number HE585565 available at www.ebi.ac.uk) was previously isolated from the rhizosphere soil of *Festuca rubra* which is a natural coloniser of the tailings in the Rubiais mine (Becerra-Castro *et al.*, 2012). It is Cd- and Zn-tolerant, and both an organic acid- and indoleacetic acid (IAA)-producer (5 mg IAA mL^{-1}). Metal resistance was determined using a minimal salt medium (with sugars and organic acids as C sources) enriched with Cd (added as $CdSO_4.8/3H_2O$ from 0 to 6 mM) or Zn (added as $Zn(SO_4)_2 \cdot 7H_2O$ from 0 to 25 mM). The Maximal Tolerable Concentration (MTC) was 4 mM Cd and 2.5 mM Zn. Organic acid production was assessed using the qualitative bromocresol method. The ability produce IAA were evaluated in liquid medium supplemented with tryptophan, and following the Salkowski method. The P30 strain did not produce siderophores and was not able to solubilise inorganic phosphate. A full description of the methods used for the phenotypic characterisation is given by Becerra-Castro *et al*. (2011). It has been shown to mobilise Zn from metal-rich minerals and soils under *in vitro* conditions (unpublished data). The strain has been previously characterised as a plant growth promoting bacteria (PGPB) due to its beneficial effect on the biomass production of a range of plant species, including *Festuca* sp., *Salix* spp. and *N. tabacum*,

when grown in metal-enriched hydroponic solutions or Cd/Zn-contaminated mine soil (Álvarez-López *et al.*, 2011; Becerra-Castro *et al.*, 2012).

A factorial study was designed to evaluate the effects of the inoculation method on biomass production and metal accumulation by *N. tabacum*. Inoculation treatments included (1) a single seed inoculation (seed) (2) a single soil inoculation (soil) (3) a soil inoculation repeated twice (dual soil) and (4) seed followed by soil inoculation (seed+soil). To test the effects of the dose of inoculum applied all four treatments were carried out using two cellular densities: 10^6 and 10^8 CFUs mL^{-1}. Non-inoculated controls (NI) were established in parallel for each treatment. Fresh cultures of the bacterial strain were grown from a glycerol stock, and then cultivated in liquid 869 medium (1.0 g tryptone, 0.5 g yeast extract, 0.5 g NaCl, 0.1 g glucose, and 0.035 g $CaCl_2.2H_2O$ in 1 L deionised water) for 24 h, after which 5 mL of this culture was used to inoculate 50 mL of 869 liquid medium. The cultures were grown overnight, harvested by centrifugation (6000 rpm, 10 min), washed and re-suspended in sterile 10mM $MgSO_4$ to two different optical densities: OD_{660} of 1.0 (about 10^8 CFUs mL^{-1}) or OD_{660} of 0.1 (about 10^6 CFUs mL^{-1}). For the seed inoculation treatment, seeds of *N. tabacum* were surface-sterilised in 1% NaClO + Tween 80 for 30 min, followed by profuse rinsing with sterile deionised water and dried on sterile filter paper. Seeds were placed in 50 mL of each of the two inocula (10^6 or 10^8 CFUs mL^{-1}) and shaken for 1 h. For the non-inoculated treatment, seeds were placed in 50 mL of 10mM $MgSO_4$. After this period, seeds were transferred into sterile jars with a 1:1 vermiculite:perlite mixture and watered with sterile half-strength Hoagland solution for one month until they were transplanted into the pots. The Hoagland solution contains per litre deionised water: 50 mL macroelements, 500 mL microelements and 300 mL Fe-EDTA (macroelements (g L^{-1}): 10.2 KNO_3, 7.08 $Ca(NO_3)_2.4H_2O$, 2.30 $NH_4H_2PO_4$, 4.9 $MgSO_4.7H_2O$; microelements (g L^{-1}): 2.86 H_3BO_3, 1.81 $MnCl_2.4H_2O$, 0.08 $CuSO_4.5H_2O$, 0.09 $H_2MoO_4.H_2O$, 0.22 $ZnSO_4.7H_2O$; Fe-EDTA (g L^{-1}): 5.00 EDTA-Na, 7.60 $FeSO_4.7H_2O$). For the soil inoculation treatment, surface-sterilised non-inoculated seeds were germinated as described above and one month-old seedlings were transplanted into the pots. One month after transplanting each plant was inoculated around the base of the stem with 10 mL of each bacterial suspension prepared as described above. The same amount of sterile 10mM $MgSO_4$ was added to non-inoculated pots. For the dual soil inoculation, a second dose of bacterial inoculum was applied one month after receiving the first dose. At the same time a second dose of sterile 10mM $MgSO_4$ was added to non-

inoculated pots. For seed+soil inoculation treatment, both single treatments were carried out (surface-sterilised inoculated seeds were germinated and one month-old seedlings were transplanted into the pots, and after one month each plant was inoculated around the base of the stem as described above). After transplantation, plant pots were placed at random in the greenhouse and grown for a further 4 months. In all cases one single seedling was planted in each pot and six replicates were prepared for each treatment and controls.

Plant and soil analyses

After 3 months, plants were harvested. Shoots and roots of plants were separated, washed with pressurised tap water followed by deionised water, oven-dried at 45 °C, weighed and ground. Oven-dried shoot tissues (0.1 g) were digested in a 2:1 mixture of concentrated HNO_3:HCl on a hot plate at 120 ºC and the concentrations of Ca, Fe, K, Mg, P, Cd, Pb and Zn were measured by ICP-OES (Vista Pro; Varian Inc., Australia). Data were expressed on the basis of dry weight (DW) plant material. Metal yield was calculated as the product of total shoot biomass produced and the shoot metal concentration. The full recovery of plant roots was not possible and only shoot dry weight was assessed. Normalised nutrient concentration values in shoots were calculated through a z score standardisation. The z score value is given by $z = (x - \mu) / \sigma$, where x is the element concentration of each sample; μ and σ are respectively the mean and the standard deviation of each element.

Soil analyses were carried out on the air-dried, < 2 mm fraction after harvesting. Changes in metal availability due to inoculation treatments were evaluated after extraction with 1M NH_4NO_3 (2 h shaking; 1:2.5 soil:extractant) and with 1M NH_4Cl (16 h equilibrium; 1:20 soil:extractant). The concentration of Cd, Pb and Zn was determined in the filtered extracts by ICP-OES (Vista Pro; Varian Inc., Australia).

Statistical analyses

Differences in plant biomass, plant and soil metal concentrations were determined using analyses of variance (one-way ANOVA). A multiple comparison of means was determined by the "post-hoc" *Tukey* test. When necessary, data were transformed to meet the requirements of ANOVA. Data which were not normally distributed were analysed using non parametric tests. Interaction effects of inoculation mode and cellular density application on the shoot biomass

production, metal concentration and metal yields were analysed through a two-way ANOVA.

7.3. Results

Effect of bacterial inoculation on biomass production

The effect of bacterial inoculation on the biomass production of *N. tabacum* was dependent on both the cellular density of the inoculum and the mode of inoculation (i.e. seed, soil, dual soil, seed+soil) (Table 7.1). Moreover, the density*mode interaction factor was statistically significant ($p < 0.01$; Table 7.1). Figure 7.1 shows the mean shoot DW of non-inoculated (NI) and inoculated plants for all four modes of inoculation and both cellular densities (10^6 and 10^8 CFUs mL^{-1}).

Table 7.1. Effect of bacterial density (density) and inoculation time (moment) on the total metal extrated (results of two-way-ANOVA). Asterisks denote statistical differences * (p < 0.05), ** (p < 0.01), * (p < 0.005)**

	df	Biomass (g)		Pb mg kg^{-1}		Zn mg kg^{-1}		Cd mg kg^{-1}	
Source		MS	*F*	MS	*F*	MS	*F*	MS	*F*
Density	1	0.152	8.64 **	0.023	0.485	0.003	0.159	0.034	1.782
Mode	3	0.163	9.23 ***	0.204	4.23 *	0.079	3.95 *	0.08	4.055 *
Density * Mode	3	0.08	4.51**	0.046	0.953	0.025	1.279	0.015	0.799
	df	Pb yield (mg)		Zn yield (mg)		Cd yield (mg)			
Source		MS	*F*	MS	*F*	MS	*F*		
Density	1	0.308	3.038	0.204	4.13	0.096	1.883		
Mode	3	0.524	5.159 **	0.191	3.85 *	0.342	6.724 ***		
Density * Mode	3	0.1	0.984	0.028	0.557	0.075	1.474		

The effect of each inoculation treatment is compared with the respective control (NI). Shoot DW of NI plants varied (from 34.0 to 38.8 g) but differences were not statistically significant ($p > 0.05$; Figure 7.1). Seed inoculation did not significantly improve shoot DW, at either cellular density (Figure 7.1). In contrast, soil inoculation (single or dual inoculation) enhanced biomass production with at

least one of the cellular densities tested (albeit not always significantly). For the single soil inoculation, mean shoot DW of non-inoculated plants was 34.6 g and increased to 50.3 and 37.6 g after addition of 10^6 or 10^8 CFUs mL^{-1}, respectively (45 and 9% increase). For the dual soil inoculation, shoot DW increased after addition of 10^6 CFUs mL^{-1} from 38.5 to 42.1 g (9%). This promotion in shoot yield was only statistically significant for the single inoculation and lower cellular density ($p < 0.05$) (Figure 7.1). Increasing the number of inoculated bacterial cells (from 10^6 CFUs mL^{-1} to 10^8 CFUs mL^{-1}) did not lead to a further improvement in biomass (Figure 7.1), and the effects of the inoculum bacterial density were not constant across all the different inoculation modes. For example, the lower cellular density of 10^6 CFUs mL^{-1} caused the highest improvement in shoot biomass production after soil inoculation; however, the same bacterial density had no significant effect on biomass production after seed or seed+soil inoculation. Likewise the higher cellular density of 10^8 CFUs mL^{-1} was found to have most effect (albeit not significant) after single soil inoculation but had no effect in the other inoculation modes. Furthermore, no significant improvement in biomass production after successive bacterial inoculation events (dual soil inoculation or seed+soil inoculation mode) was found compared to a single inoculation event (Figure 7.1).

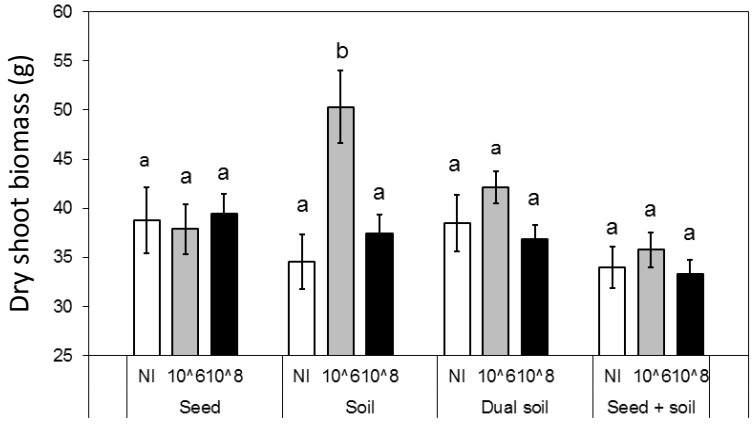


Figure 7.1. Effects of the different inoculation treatments on the mean shoot dry weight of *N. tabacum* (mean ± SE). Different letters above bars indicate significant differences ($p < 0.05$) within each treatment (NI, 10^6 CFUs mL^{-1} and 10^8 CFUs mL^{-1}) (n=6).

Shoot metal concentrations and effect of bacterial inoculation on shoot ionome and metal accumulation

Shoot metal concentrations in non-inoculated plants varied from 12.7 mg kg^{-1} to 14.9 mg kg^{-1} for Cd, from 208.9 to 239.4 mg kg^{-1} for Zn, and from 5.0 mg kg^{-1} to 7.6 mg kg^{-1} for Pb (Figure 7.2). Metal concentrations did not differ significantly amongst the NI plants, except for Pb (which was significantly lower in the case of NI plants of the single soil inoculation mode ($p < 0.05$; Figure 7.2).

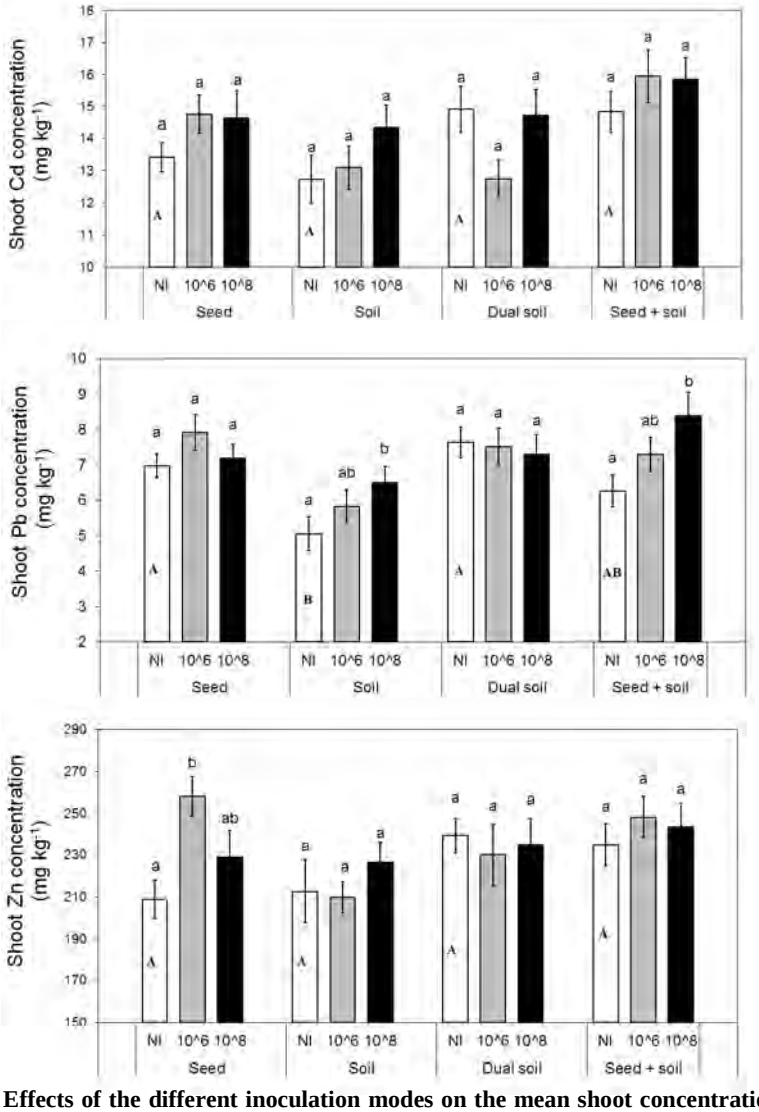


Figure 7.2. Effects of the different inoculation modes on the mean shoot concentration of Pb, Zn and Cd (mean ± SE). Different letters above bars indicate significant differences ($p < 0.05$) within each treatment (NI, 10^6 CFUs mL^{-1} and 10^8 CFUs mL^{-1}) (n=6). Capital letters within bars indicate differences between NI treatments ($p < 0.05$).

In general, bacterial inoculation did not significantly affect the plant nutrient status and only a few significant differences were found for Fe, K and P (Table 7.2). Seed inoculation had no effect on the shoot concentrations of Ca, P, Mg or K, but a slight but significant decrease in Fe was observed after inoculating with a bacterial density of 10^8 CFUs mL^{-1} (from 0.25 g kg^{-1} to 0.20 g kg^{-1}; $p < 0.05$). Soil inoculation tended to increase the shoot Ca, K, Mg and P concentrations, with both cellular densities. However, this effect was only significant for P (10^6 CFUs mL^{-1}) and for K (10^8 CFUs mL^{-1}) ($p < 0.05$). After dual soil inoculation, nutrient concentrations tended to decrease for all elements except K: using a bacterial density of 10^6 CFUs mL^{-1} the shoot K concentration increased from 45.5 mg kg^{-1} to 49.3 mg kg^{-1}, albeit not statistically significant. The decrease in nutrient concentrations was statistically significant in the case of Fe with both bacterial densities (from 0.29 g kg^{-1} to 0.20 g kg^{-1} and of P (from 3.38 g kg^{-1} to 2.88 g kg^{-1} (10^8 CFUs mL^{-1})). After seed+soil inoculation shoot K and P concentrations tended to be higher in inoculated plants (only significant in the case of 10^8 CFUs mL^{-1}; $p < 0.05$): P concentration increased from 2.37 g kg^{-1} to 2.80 g kg^{-1} and K from 40.5 g kg^{-1} to 46.7 g kg^{-1}.

Table 7.2. Concentrations of major nutrients in tobacco shoots (mean ± SE) Different letters indicate significant differences ($p < 0.05$) within each treatment (NI, 10^6 CFUs mL^{-1} and 10^8 CFUs mL^{-1}) (n=6).

		Ca (g kg^{-1})	Fe (g kg^{-1})	K (g kg^{-1})	Mg (g kg^{-1})	P (g kg^{-1})
Seed	NI	19.2 ± 1.1 a	0.25 ± 0.02 b	44.9 ± 1.1 a	3.74 ± 0.16 a	2.56 ± 0.11 a
	10^6	19.4 ± 0.8 a	0.21 ± 0.01 b	41.9 ± 0.5 a	3.57 ± 0.13 a	2.55 ± 0.08 a
	10^8	19.7 ± 0.8 a	0.20 ± 0.01 a	44.4 ± 1.1 a	3.73 ± 0.19 a	2.70 ± 0.09 a
Soil	NI	16.4 ± 1.1 a	0.17 ± 0.02 a	37.6 ± 0.8 a	3.21 ± 0.10 a	2.24 ± 0.06 a
	10^6	18.8 ± 1.0 a	0.17 ± 0.01 a	39.3 ± 0.8 a	3.37 ± 0.09 a	2.71 ± 0.06 b
	10^8	18.6 ± 0.8 a	0.20 ± 0.02 a	39.4 ± 0.9 b	3.40 ± 0.13 a	2.57 ± 0.08 a
Dual soil	NI	22.0 ± 0.8 a	0.29 ± 0.02 b	45.5 ± 1.3 a	4.09 ± 0.10 a	3.38 ± 0.10 b
	10^6	19.6 ± 1.1 a	0.20 ± 0.02 a	49.3 ± 1.5 a	3.81 ± 0.12 a	2.90 ± 0.12 ab
	10^8	20.0 ± 0.9 a	0.20 ± 0.01 a	44.5 ± 1.4 a	3.88 ± 0.15 a	2.88 ± 0.10 a
Seed+soil	NI	18.0 ± 0.9 a	0.20 ± 0.02 a	40.5 ± 1.4 a	3.46 ± 0.19 a	2.37 ± 0.05 a
	10^6	18.1 ± 1.1 a	0.20 ± 0.01 a	44.4 ± 1.4 ab	3.45 ± 0.09 a	2.54 ± 0.11 ab
	10^8	16.8 ± 0.4 a	0.22 ± 0.02 a	46.7 ± 1.2 b	3.46 ± 0.16 a	2.80 ± 0.08 b

The z scores calculated for each nutrient indicated that the shoot element concentrations grouped differently according to treatments (Figure 7.3). Although not consistent, a general positive effect on shoot nutrient concentrations was found in those treatments in which soil was inoculated once (soil and seed+soil treatments). However, the addition of $MgSO_4$ (non-inoculated treatments) also had an effect on the shoot metal concentrations: the respective controls of the seed and dual soil inoculation treatments presented the highest concentrations of K and Fe or of Ca, K and P in tobacco shoots, respectively.

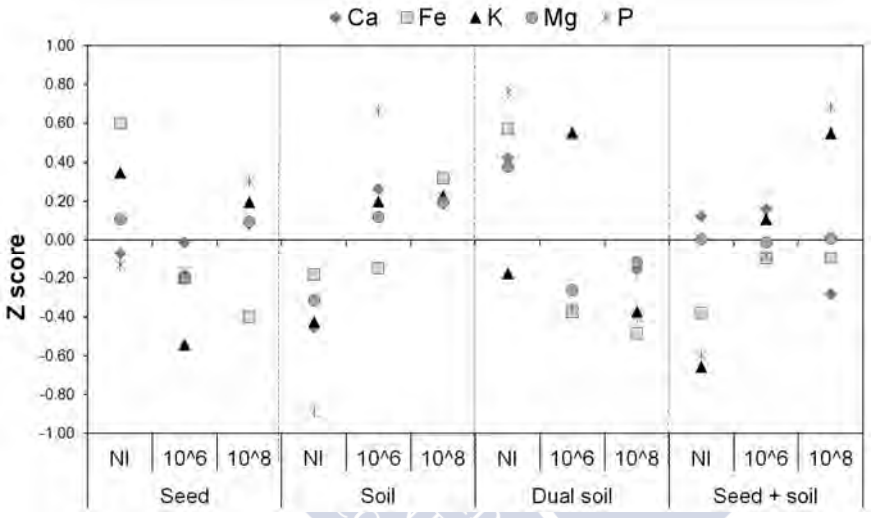


Figure 7.3. Mean values of z-scores performed in relation to concentrations of Ca, Fe, K, Mg, and P in shoots of *N. tabacum* growing in the different treatments. The z score value is given by $z = (x - \mu) / \sigma$, where x is the element concentration of each sample; μ and σ are respectively the mean and the standard deviation of each element.

The inoculation mode, but not the inoculum density, significantly influenced shoot metal concentration ($p < 0.05$) compared to corresponding NI plants ($p < 0.05$; Table 7.1 and Figure 7.2). Shoot Pb concentration increased (albeit not significantly) compared to non-inoculated plants after addition of 10^6 CFUs mL^{-1} in the seed, soil, and seed+soil inoculation modes (by 13%, 15% and 17%, respectively). Addition of 10^8 CFUs mL^{-1}, led to a significant increase in the shoot Pb concentration ($p < 0.05$) in the soil inoculation (from 12.6 mg kg^{-1} in NI plants to 16.2 mg kg^{-1} in inoculated plants) and seed+soil inoculation modes (from 15.6 mg kg^{-1} in NI plants to 20.9 mg kg^{-1} in inoculated plants). In contrast to what was observed for biomass, successive inoculations (seed+soil inoculation) increased the mean shoot Pb concentration and this was observed with both cellular densities. In the case of the shoot Zn concentration, a significant increase ($p <$

0.05) was observed after seed inoculation with the lower cellular density (from 522.1 mg kg^{-1} in NI to 645.1 mg kg^{-1} in inoculated plants) and also a tendency to increase shoot Zn concentration was found with the higher cellular density (by 10%). A slight but not significant increase in shoot Zn concentration was observed after inoculation with the bacterial density of 10^8 CFUs mL^{-1} for the soil inoculation mode (7%) and after inoculation with the bacterial density of 10^6 CFUs mL^{-1} for the seed+soil inoculation mode (6%). No significant differences in shoot Cd concentration were observed, although a tendency towards a higher concentration was observed with both bacterial densities after seed (10% and 9%) and seed + plant inoculation (8% and 7%), and with the density of 10^8 CFUs mL^{-1} after soil inoculation (13%).

Effect of bacterial inoculation on metal availability in soils

The effect of plant growth and bacterial inoculation on soil labile metal pools were evaluated using NH_4NO_3 and NH_4Cl extractants (Figure 7.4). After 16 weeks of growth the labile (NH_4Cl- and NH_4NO_3-extractable) Zn pool was reduced by up to 67% and 51% (in non-inoculated *N. tabacum*) which was likely due to plant uptake and accumulation (Figure 7.4). NI plants also decreased available Cd by up to 58% and 74%, (NH_4Cl- and NH_4NO_3-extractable, respectively) (Figure 7.4). Inoculation treatments also affected the soil metal availability (albeit not always significantly). Moreover, the same effects on metal availability

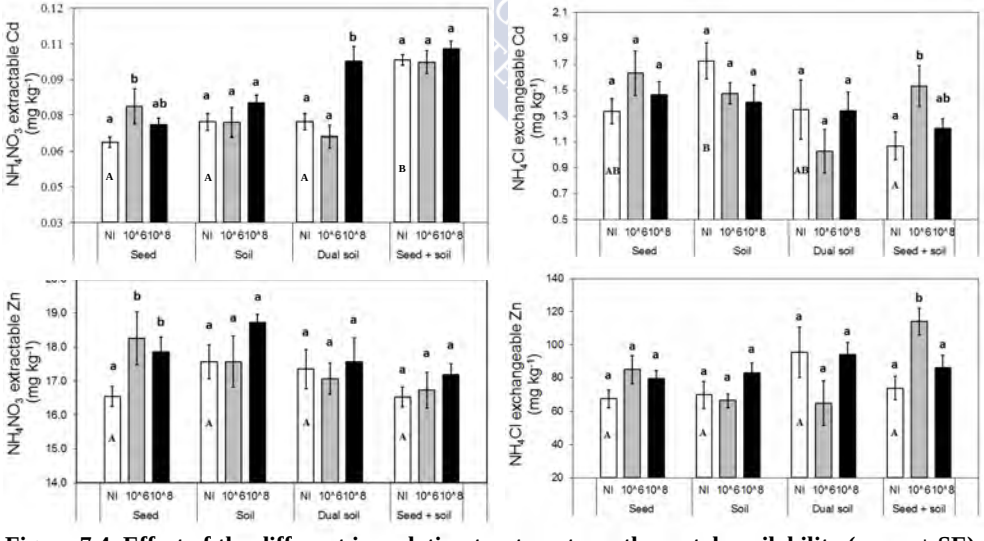


Figure 7.4. Effect of the different inoculation treatments on the metal availability (mean ± SE) assessed using NH_4NO_3 and NH_4Cl soil extractions. Different letters above bars indicate significant differences ($p < 0.05$) within each treatment (NI, 10^6 CFUs mL^{-1} and 10^8 CFUs mL^{-1}) (n=6). Capital letters within bars indicate differences between NI treatments ($p < 0.05$).

were not always observed with both soil extractants, and the main divergences between the two extractants were observed in the seed+soil treatment. The concentrations of NH_4NO_3-extractable Zn and Cd in pots after seed inoculation with a bacterial density of 10^6 CFUs mL^{-1}, were significantly higher than in NI plants (from 16.5 to 18.3 mg Zn kg^{-1} and from 0.06 to 0.08 mg Cd kg^{-1} respectively; $p < 0.05$). Similarly, a higher Zn and Cd availability measured with NH_4NO_3 (only significant for Zn) was found after inoculation with the higher bacterial density (from 16.5 to 17.6 mg Zn kg^{-1}; $p < 0.05$). The single and dual soil inoculation with the higher bacterial density of 10^8 CFUs mL^{-1} tended to increase NH_4NO_3-extractable Zn and Cd but the difference detected was only significant for Cd after dual soil inoculation (0.07 mg kg^{-1} to 0.10 mg kg^{-1}, $p < 0.05$). Similar tendencies were observed using the NH_4Cl extractant but the differences were not significant. Conversely, in the case of seed+soil inoculations, changes in Zn or Cd availability were not detected with NH_4NO_3; but the NH_4Cl-extractable Zn and Cd concentrations were significantly higher in pots receiving inocula of 10^6 CFUs mL^{-1} (113.9 mg Zn kg^{-1} and 1.53 mg Cd kg^{-1}) than in NI pots (73.8 mg Zn kg^{-1} and 1.07 mg Cd kg^{-1}).

Effect of bacterial inoculation on plant metal yield

The efficiency and success of metal phytoextraction depends on the total amount of extracted metal (or metal yield) which is dependent on the total amount of biomass produced and the accumulated metal in harvestable tissues. Figure 7.5 presents the percentage increase or decrease in plant metal yields for the four inoculation modes and cellular densities in relation to NI plants; metal yield was only significantly infuenced by the inoculation mode (Table 7.1) ($p < 0.05$). Interaction effects did not reach statistical significance for metal (Table 7.1; $p > 0.05$).

Non-inoculated plants extracted between 0.18 to 0.30 mg Pb $plant^{-1}$, 7.08 to 9.18 mg Zn $plant^{-1}$ and 0.43 to 0.57 mg Cd $plant^{-1}$. In general, all the treatments led to an improvement in the phytoextraction of the evaluated metals and the most pronounced effects were found for Pb. However, the single soil inoculation mode was the only case where a significant increase in the yield of all three metals was found using both celullar densities. The best results in this inoculation mode were achieved using the lower bacterial density, which caused increases in Pb, Zn and Cd yields of up to 67%, 49% and 57%, respectively ($p < 0.05$; Figure 7.5). Finally, the dual soil inoculation using 10^8 CFUs mL^{-1} tended to reduce the amount of

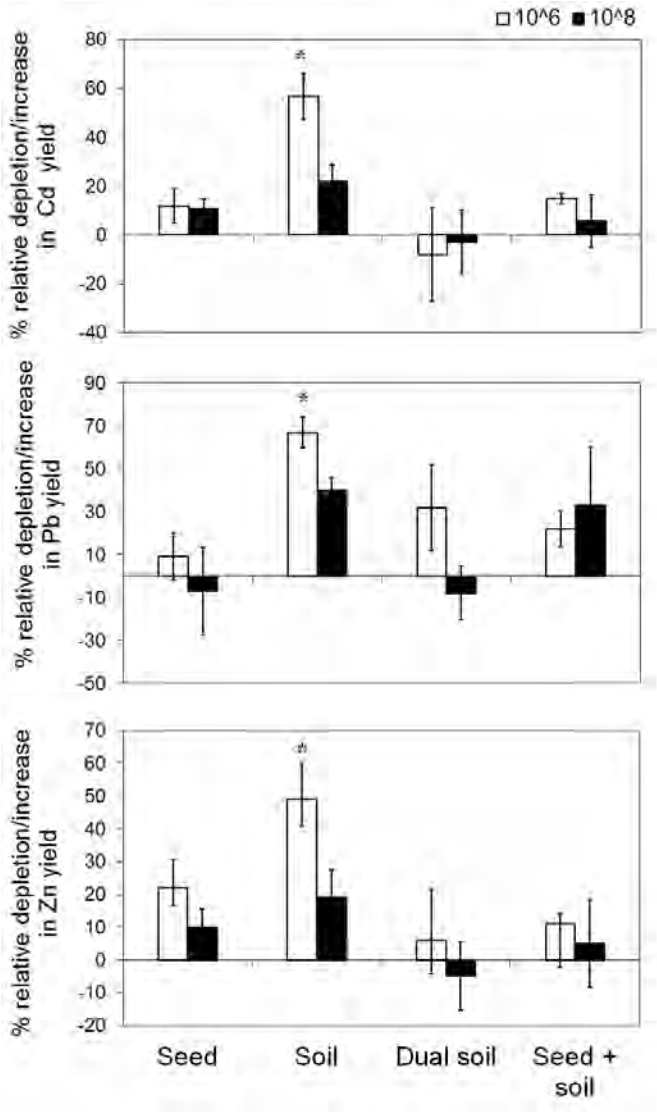


Figure 7.5. Percentage of increase / depletion in total metal extracted (metal yield) relative to the NI treatment (n = 6). The depletion / increase was calculated by substracting the amount of accumulated metals in the NI plants from the corresponding amounts in each inoculation treatment (mean ± SE). A significant depletion / increase is denoted by an asterisk ($p < 0.05$).

extracted metal for all three elements (Pb, Zn and Cd), and Cd extraction was also reduced in this treatment when using 10^6 CFUs mL^{-1}.

7.4. Discussion

The PGP effect of strain P30 was found to be dependent on the inoculation method and cellular density of the inoculum. The selected inoculation methods used (seed and soil inoculation) represent the most common practices used for the application of microbes to soil (Afzal *et al.*, 2011). Seed inoculation has been integrated within phytoremediation techniques targeting organic- (Germaine *et al.*, 2009; Gurska *et al.*, 2009) or metal-contaminated sites (Kolbas *et al.*, 2015). Bashan (1986) tested the effect of inoculation time (seed and soil inoculation) on bacterial induced effects on plant growth, and found that seed inoculation showed the best results in terms of increasing shoot DW. In contrast, in this study, soil inoculation (inoculation after plant emergence) achieved the best results in terms of plant growth promotion. Similarly, Afzal *et al.* (2011) found that bacterial strains applied by soil inoculation significantly enhanced hydrocarbon degradation and rhizosphere colonisation to a higher degree than when the strains were applied to seeds. These authors observed a poorer colonisation after seed inoculation and attributed this to the differences in the exudates released which favoured the growth of other microorganisms already established on the seed coat. It has been shown that a poor microbial survival in the rhizosphere can led to a reduction in the desirable positive effects (Gilbertson *et al.*, 2007; Gunderson *et al.*, 2007).

Both bacterial densities used for the inoculations in this study are within the ranges of those commonly used in the literature (Kuffner *et al.*, 2010; Marques *et al.*, 2013; Petriccione *et al.*, 2013; Langella *et al.*, 2014). Increasing the number of inoculated bacterial cells (from 10^6 CFUs mL^{-1} to 10^8 CFUs mL^{-1}) did not lead to a further improvement in biomass. Moreover, increasing the number of inoculation events could be considered a crucial factor influencing the success of establishment and colonisation of the desired bacterial strain in the rhizosphere, but this aspect has rarely been studied in phytoremediation-orientated studies. Here we did not find an improvement in biomass production after successive bacterial inoculation events (dual soil or seed+soil inoculations) and none of these increased plant growth by more than 10%. Bashan (1986) also showed that multiple inoculations have a marginal effect on plant growth, and only after four successive inoculations was an increase in plant response observed. These authors attributed this effect to poor root colonisation. The less pronounced effect after a second soil

inoculation could be related to the presence of a more established bacterial community in the rhizosphere of tobacco plants which impedes the possibilities of the inoculum at the later plant growth stage to colonise efficiently. Whereas at the first inoculation event newly transplanted and rapidly growing plants allow for the inoculum to establish and proliferate more effectively.

Tobacco plants have been previously described as efficient metal phytoextractors (Fässler *et al.*, 2010; Guadagnini, 2000). In the present study, the shoot Cd and Zn concentrations were close to the upper threshold values of the phytotoxic concentrations for these elements (5-30 mg kg^{-1} Cd; 100-400 mg kg^{-1} Zn) as proposed by Kabata-Pendias (2010). On the other hand, shoot Pb concentrations were well below the 30-300 mg kg^{-1} phytotoxic concentration threshold given for this element. A similar range in shoot concentrations of Cd (2.5-25 mg kg^{-1}), Zn (150-850 mg kg^{-1}) and Pb (15-30 mg kg^{-1}) were observed in tobacco variants assessed in experimental metal-contaminated field sites in Balen (Belgium) and Rafz (Switzerland) (PHYTAC, 2005). Both these sites are agricultural soils which were affected by fall-out from a Zn/Pb smelter (Balen) or by sewage sludge application (Rafz). The present study suggests that tobacco is also a potential candidate for cultivation in contaminated mine-soils, although field assessments would be required to determine its full potential.

The efficiency and success of metal phytoextraction depends on the total amount of extracted metal (or metal yield) which is dependent on the total amount of biomass produced and the accumulated metal in harvestable tissues. Depending on the inoculation method and cellular density, strain P30 induced an increase in the biomass production of *N. tabacum*. This strain is an IAA-producer and the beneficial effects of this phytohormone on plant growth are well known (Dell'Amico et al. 2008; Duca *et al.*, 2014). Inoculation with strain P30 resulted in a more successful growth promotion (up to 45% increase) than metal accumulation enhancement (up to 34% increase) compared to non-inoculated plants. This result coincides with other studies in which the bacterial inoculants used tended to be more successful in promoting plant growth, rather than increasing shoot metal concentration. For example, Janssen *et al.* (2015) observed an increase in Zn/Cd phytoextraction efficiency of *S. viminalis* by increasing twigs growth after inoculation with *Rahnella* sp. an IAA-, ACC deaminase-, organic acids- and siderophore- producer. Lampis *et al.* (2015) also found than a bacterial inoculum combination of siderophore- and IAA- producers (*Bacillus sp. MPV12, Variovorax sp. P4III4, and Pseudoxanthomonas sp. P4V6*) increased biomass of

Pteris vitatta by up to 30% increasing total As removal. Kuffner *et al.* (2010) observed an increase in growth of willows after inoculation with the strain *Agromyces* AR33. Sessitsch *et al.* (2013) carried out a meta-analysis of phytoremediation-orientated inoculation studies (in which over 70 publications and 738 individual cases or treatments were included) and found that the most frequent effects observed of plant inoculation were an increase in shoot biomass (30% of cases). Although there was no significant increase in the shoot Cd concentration of *N. tabacum* after soil inoculation with strain P30, the increase in shoot DW biomass led to a significant increase in the Cd removal. This strain could therefore be a potential candidate for improving the metal extraction capacity of phytoextracting plants. Similarly, Becerra-Castro *et al.* (2012) observed that rhizosphere bacterial strains did not affect Cd and Zn accumulation in the leaves of *Salix viminalis* but the microbial-induced increase in biomass production resulted in an increase in the amount of trace elements phytoextracted. In the present study, positive effects of bacterial inoculation on metal uptake by tobacco were also observed (especially Pb). An increase in metal uptake by tobacco after bacterial inoculation was also reported by Mastretta *et al*. (2009), who found that the inoculation of *Nicotiana tabacum* seeds with the endophyte *Sanguibacter* sp. Sd2 increased shoot Cd concentrations compared to non-inoculated plants. Ma *et al*. (2015) also found an increase in Cd and Zn uptake by *Sedum plumbizicola* after inoculation with *B. pumilus* E2S2 and *Bacillus* sp E1S2 and Sangthong *et al*. (2015) observed that bacterial inoculation enhanced Cd shoot concentration in maize plants. After 12 weeks of growth the labile (NH_4Cl-extractable) Zn pool was reduced by up to 33% (in non-inoculated *N. tabacum*) which was likely due to plant uptake and accumulation. Likewise, Herzig *et al*. (2014) found a decrease in the labile ($NaNO_3$-extractable) Zn pool of up to 69% in the soils at Rafz planted with the same variant (NBCu 10-8, 1st generation).

Overall, no significant correlation was found between the abundance of inoculated bacteria and shoot DW, metal concentration or removal. These results are in agreement with other studies in which the authors evaluated the effects of increasing the bacterial density of inocula (Bashan, 1986; Rai *et al.*, 2004; Braud *et al.*, 2006; Ramírez and Kloepper, 2010). Similar findings have been obtained when using mycorrhizal inocula, since results indicate that plant growth is not correlated with the rate of mycorrhizal colonisation (Baum *et al.*, 2006). Our results therefore indicate that the activity of the inoculated bacterial strain in the soil could be more or equally as important as the cellular density of the inoculum.

Schwartz *et al*. (2000) attributed the more beneficial effects of a lower bacterial density in plant growth promotion to the depletion of limited soil resources necessary for growth and bacterial survival when a higher density is inoculated. In our case, bacterial inoculation using the lower cellular density (10^6 CFUs mL^{-1}) actually had a more pronounced growth promoting effect than the inoculum with a higher cellular density (10^8 CFUs mL^{-1}). A high dose of inoculum might also have a deleterious effect on other soil microorganisms which are beneficial for tobacco growth. Similar results were found by Kapulnik *et al*. (1985) who showed that plant response to high levels of *Azospirillum* was negative and that the optimal bacterial level of inoculation in hydroponic systems was 10^5 - 10^6 CFUs mL^{-1}.

7.5. Conclusions

Several studies can be found on the development of PGPB inoculation methods (principally for N_2-fixing bacteria) for agricultural food crops. In contrast, the optimisation of inoculation methods for plants growing in contaminated soils has rarely been assessed, and even less so in phytoextraction-orientated studies. In general, inoculation with the IAA-producing bacterial P30 strain positively affected the phytoextraction process of the three problematic metals (Pb, Zn and Cd) in the mine soil. The most pronounced effect was observed in plant biomass production, and to a lesser extent in the shoot metal accumulation and plant nutritive status. Our results indicated that both the inoculation mode and the cellular density of the inoculum are important factors to take into account when designing inoculation strategies, since they can modify the obtained results in terms of plant performance and soil metal removal. In contrast to what was expected, a single soil inoculation event was found to give the best results together with the lower bacterial density (10^6 CFUs mL^{-1}). Further studies at a field scale will be required to corroborate the beneficial effects of the best inoculation method selected in this study.

7.6. References

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Chapter 8

Monitoring the effectiveness of (aided) phytostabilisation implemented in Cu-rich mine tailings

ABSTRACT

(Aided) phytostabilisation has been proposed as a suitable technique to decrease the environmental risks associated with metal(loid)-enriched mine tailings. It combines trace element (TE)-excluding plants and soil amendments to promote the *in situ* inactivation of metal(loid)s and establish a vegetation cover. The use of organic waste-based amendments can modify TE mobility, provide micro- and macro-nutrients for plant growth, and improve soil physical properties, water infiltration and water holding capacity, thereby enhancing soil fertility, organic matter accumulation and soil functionality. A field trial of aided phytostabilisation was implemented in spring 2011 in Cu-rich mine tailings in the NW of Spain. The tailings were amended with composted municipal solid wastes and planted with *Salix* spp., *Populus nigra* L. or *Agrostis capillaris* L. cv. Highland. Plant growth, nutritive status and metal accumulation, and soil physico- and bio-chemical properties, were monitored over three years. Compost amendment improved soil properties such as pH, CEC and fertility, and decreased soil Cu availability, leading to the establishment of a healthy vegetation cover. Aided phytostabilisation stimulated microbial activity and led to the establishment of vital biogeochemical cycles. *S. viminalis* and *A. capillaris*, showed the best results in terms of plant growth and biomass production. The main effects on soil physicochemical parameters were a direct result of compost addition, while plant-induced effects were observed in biochemical parameters. The beneficial effects were maintained at least three years after treatment.

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8.1. Introduction

Mining activities are one of the main sources of anthropogenic metallic contamination worldwide, and mine tailings or spoils are often characterised by a lack of organic matter (OM) and nutrients, a high acidity, limited water holding capacity (WHC) and potentially toxic concentrations of trace elements (TE) (Wong, 2003). Phytomanagement has been proposed as a suitable technique to decrease the environmental risks associated with metal(loid)-enriched mine tailings (Conesa *et al.*, 2012). Phytostabilisation aims to establish a vegetation cover and progressively promote the *in situ* inactivation of trace elements by use of TE-excluding plants and/or soil amendments. When soil amendments are used the process is often referred to as (aided) phytostabilisation (Mench *et al.*, 2006; Vangronsveld *et al.*, 1995, 2009). This type of phytomanagement has been shown to reduce TE mobility by altering speciation, as well as to improve soil physico-chemical properties and fertility (Clemente *et al.*,2012; Zornoza *et al.*, 2012; Pardo *et al.*,2013). Moreover, it can lead to an increase in microbial diversity and restore functionality of TE-contaminated sites in the long term (Renella *et al.*, 2008; Kumpiene *et al.*,2009; Xue *et al.*, 2015).

In Europe, numerous grass species have been proven effective in establishing long-term plant cover in heavily-contaminated sites, namely *Poa pratensis*, *Agrostis capillaris*, *Festuca arundinacea*, *Festuca rubra* and *Festuca ovina* (Stuczynski *et al.*,2007). Several authors have also proposed incorporating short rotation coppice (SRC) systems with woody crops (such as *Salix* and *Populus*) into the phytomanagement system (Pierzynski *et al.*, 2002; Pulford and Dickinson, 2006; Witters *et al.*, 2009). These tree species are well suited for phytostabilisation of several metal(loid)s (e.g. Cu and Pb) due to their deep root systems, tolerance to TE and nutrient deficiency, and the ability to re-sprout from the stumps after harvests (normally performed at short time intervals, i.e. 2 – 6 years) (Dimitriou *et al.*, 2012). Moreover, the harvested biomass can be used for bioenergy purposes, timber production or the feedstock for pyrolysis or biofortified products (Witters *et al.*, 2009; Conesa *et al.*, 2012; Chalot *et al.*, 2012).

Soil amendments like liming agents, phosphates and apatites, Fe, Al and Mn (oxyhydr)oxides, organic amendments, and industrial waste products have been widely used in phytostabilisation experiments. The formation of insoluble, sorbed, or bound chemical species of TE reduces leaching through the soil profile and the labile metal(loid) pool in the soil (Vangronsveld *et al.*, 1995; Mench *et al.*, 2003;

Clemente *et al.,* 2006; Ruttens *et al.,* 2006). Incorporation of organic residues (in particular composts, manure, sewage sludge or biosolids) improves soil physical properties, water infiltration and WHC. They further contain micro- and macro-nutrients for plant growth, and also decrease bulk density (Vangronsveld *et al.,* 2009; Soriano-Disla *et al.,* 2010). Addition of this type of amendment is a common practice to facilitate re-vegetation of contaminated soils and at the same time provides a viable manner of recycling waste products. Zanuzzi *et al.* (2009) amended acidic mine tailings with pig manure or sewage sludge in combination with blanket application of marble wastes. Two years after soil amendment soil pH increased (from pH 2.7 to 7.4) alongside a build-up in total organic carbon. The authors proposed that the formation of stable soil organic matter–calcite complexes provided water and nutrients to support initial seedling establishment in the mine tailings. After 5 years, microbial biomass and soil enzymatic activities, as well as vegetation cover and richness, were higher in the amended soils than in the untreated mine tailings (Zornoza *et al.,* 2012).

The effects of organic amendments on TE bioavailability depend on the OM nature, and on the particular soil type and elements concerned (Clemente *et al.,* 2005; Ruttens *et al.,* 2006; Kumpiene *et al.,* 2011; Lagomarsino *et al.,* 2011). Some forms of unstabilised organic matter such as manure or biosolids may increase TE mobility due to their high dissolved organic matter (DOM) content that can complex metals and result in their leaching through soil (Kiikkilä *et al.,* 2002a; Tandy *et al.,* 2009). Composting stabilises organic wastes and can reduce their DOM content and the potential for metal(loid) leaching (Kiikkilä *et al.,* 2002b). Composted organic wastes can successfully improve the quality and reduce the mobility of contaminants in metal-contaminated mine-soils (Pardo *et al.,* 2011; Solís-Dominguez *et al.,* 2012; Novo *et al.,* 2013).

Only a limited number of studies evaluating the feasibility of phytostabilisation techniques under field conditions can be found, and there is a clear need for long-term field experiments monitoring the efficiency of these processes if they are to reach full-scale deployment (Mench *et al.,* 2009, 2010; Vangronsveld *et al.,* 2009; Kidd *et al.,* 2015). Spain has a long history of mining activities as a result of the large extensions of metalliferous ore deposits and is one of the primary producers of Cu, Fe, Zn and Pb in the European Union (Instituto Geológico y Minero de España, 2014). Here, a field trial of aided phytostabilisation was implemented in spring 2011 in Cu-rich mine tailings in the NW of Spain. The mine tailings at this site were amended with composted municipal solid wastes and planted with

Salix spp., *Populus nigra* L. or *Agrostis capillaris* L. cv. Highland. Plant growth, nutritive status and metal accumulation, and soil physico-chemical and biological properties, were monitored over four years. The objective was to establish a healthy plant cover (either a SRC system or a grass cover) by reducing Cu mobility and phytotoxicity and improving soil fertility and health.

8.2. Materials and Methods

Study site

Experimental field plots were established in spring 2011 in tailings at the Touro copper mine (A Coruña (NW Spain); 42º52'34"N, 8º20'40"W). The mine was active from 1974 until 1988 and the area is now confined to extraction of material for road construction. The geological substrate and characteristics of the tailings were previously described by Calvo de Anta *et al.* (1991), Vega *et al.* (2006), Álvarez *et al.* (2011) and Asensio *et al.* (2013). The mine tailings cover an area of around 550 ha and are practically bare of vegetation. The geological substrate is amphibolite, with significant quantities of metal sulphides (pyrite, pyrrhotite, and chalcopyrite). The mine soils are shallow and extremely gravelly, and are classified as Spolic Technosols (Episkeletic) (IUSS Working Group WRB, 2006). The uncontrolled oxidation of sulphides generates hyperacidic (pH 2-3) and hyperoxidising (>500 mvol) soils and waters, with a high electrical conductivity (EC) and high concentrations of sulphates, Fe, Al, and potentially toxic trace metal(loid)s. The area selected for the experimental field plots was completely barren of vegetation. The climate of the region is Atlantic (oceanic), with a mean annual precipitation of 1900 mm and mean annual temperature of 12.6ºC (Asensio *et al.*, 2013).

Experimental design and set-up

The experimental field plot (surface area of approx. 0.1 ha) was amended with mature organic compost in spring 2011 at an addition rate of 250 kg Mg^{-1} dry tailings. The compost was elaborated from municipal solid wastes and bark chippings by the local company Tratamientos Ecológicos de Noroeste (TEN s.l.) which is dedicated to the recycling of waste products and manufacturing of organic fertilisers and soil improvers. The physicochemical properties of the compost are given in Table 8.1. The tailings (the first 50 cm of surface soil) were broken-up prior to compost addition using a bull dozer (equipped with ripper),

and mechanical soil tillage was carried out after compost addition to a depth of 0-30 cm. The compost was mechanically applied.

Three replicate sub-plots (5 x 5 m) were planted four weeks after amendment incorporation with each plant species. *A. capillaris* cv. Highland was seeded at a rate equivalent to 250 kg ha^{-1}. Rooted cuttings of two *Salix* spp. (*Salix caprea* Mauerbach (BOKU 01 AT-004) and *S. viminalis*) and *P. nigra* were planted with a 1 m spacing to yield a planting density of 10000 cuttings ha^{-1}. The cuttings of *S. viminalis* were obtained from the Touro mine itself where they can be found growing sporadically. In hydroponic solutions they showed relevant metal tolerance and reduced transport of metals (including Cu) to aerial tissues (unpublished results). *Salix caprea* Mauerbach was shown in previous experiments to be metal tolerant and exclude Cu from its aerial shoots (Dos Santos Utmazian *et al.*, 2007). Cuttings of *P. nigra* were obtained from the Biogeco experimental site at a former wood preservative facility (Gironde, France) where phytostabilisation trials were implemented in 2006 to reduce Cu mobility and phytotoxicity (Lagomarsimo *et al.*, 2011). Unplanted compost-amended sub-plots (5 x 5 m) were also established to evaluate the effects of plant growth and activity. Non-amended plots were not established since nothing grows in the mine tailings unless some type of amendment is added. Weed control was carried out mechanically throughout each growth season over the period 2011 to 2015. The set-up of the experimental plots, incorporation of the compost amendment and evolution in plant growth can be seen in Figure 8.1.

Soil sampling and analyses

Soil samples (five samples per sub-plot from the upper 20 cm layer) were taken with an unpainted stainless steel spade to determine the baseline trace metal content and general physicochemical properties across the whole experimental plot before amendment addition/planting (T=0). To monitor changes in the physicochemical, biochemical and biological properties soils were then sampled 1, 2 and 3 years after incorporating the compost. Each soil sample was divided into two parts, one part for physicochemical analyses and one part for biochemical/ microbiological analyses. For physicochemical analyses, soil samples were air-dried and sieved to <2 mm. Fresh soil samples were sieved to <2 mm and stored at 4ºC for biochemical analyses or at -60ºC until DNA extraction for microbiological analyses.

Soil pH was measured in H_2O and 0.1 M KCl using a 1:2.5 (w/v) ratio. Total C and N were analysed by combustion with a CHN analyser (Model CHN-1000,



Figure 8.1. Implementation of the aided phytostabilisation field trial in the mine tailings. (a) Non-amended mine tailings, (b, c) addition and incorporation of compost amendment, (d) planting of woody tree cuttings, (e) growth of woody species after 1 year, (f) growth of *Agrostis capillaris* after 2 years, (g, h) growth of *Salix* and *Populus* after 2 years and (i) after 3 years, and (j-m) re-sprouting of trees after the first harvest.

LECO Corp., St Joseph, MI). Soils were extracted with Olsen´s reagent (0.5 M $NaHCO_3$ adjusted to pH 8.2, 1:20 w/v), and available P determined colorimetrically using the molybdenum blue method (Murphy and Riley, 1962). Soils (0.5 g) were digested in a 3:1 mixture of concentrated HNO_3:HCl after the addition of 3 mL H_2O_2 in Teflon PFA vessels in a microwave accelerated reaction system (MarsXpress; CEM Corp., USA) and the total concentrations of metals were analysed by inductively coupled plasma optical emission spectrometry (ICP-OES, model Vista-PRO, Varian). A certified soil standard (ISE 979 Rendzina Soil) was used to ensure quality of metal quantification. Exchangeable cations (Ca, Mg, Al, Na and K) were extracted with 1M NH_4Cl (1:20 w/v 16h equilibration) and measured by ICP-OES. Metal availability was evaluated by extraction with 0.1 M $NaNO_3$ (1:2.5 w/v, 2h shaking) and elements were analysed by ICP-OES.

Soil biochemical properties were monitored in collaboration with the Soil Enzymology Group of the IIAG (CSIC). Soil enzymatic analyses were determined in the non-amended tailings soil (before incorporation of compost), non-planted compost-amended soil and the compost-amended soil with either *S. viminalis* or *A. capillaris* plant cover after 1, 2 and 3 years. Dehydrogenase activity was determined as described by Camiña *et al.* (1998), and the activity was ex-

expressed as µmol INTF g^{-1} h^{-1}. Catalase activity was determined according to Trasar-Cepeda *et al.* (1999) and expressed in mmol H_2O_2 consumed g^{-1} h^{-1}. Urease activity was determined as described by Nannipieri *et al.* (1980), except that the released NH_4^+ was measured with an ammonia electrode. The activity was expressed in µmol NH_3 g^{-1} h^{-1}. Invertase activity was determined following the method described by Schinner and von Mersi (1990). Acid and alkaline phosphomonoesterase activity were determined at pH 5.0 and 10.0, respectively, with 16 mM *p*-nitrophenyl phosphate as a substrate following the method of Tabatabai and Bremner (1969), but with some modifications. Modified Universal Buffer was used to maintain the pH of the reaction mixture as described by Trasar-Cepeda *et al.* (1985). After the incubation (30 min) 2 M $CaCl_2$ was added (to prevent dispersion of soil colloids and to avoid the brown coloration caused by organic matter) and the liberated *p*-nitrophenol was extracted with 0.2 M NaOH. The enzymatic activity was quantified by reference to calibration curves corresponding to *p*-nitrophenol standards incubated with each soil under the same conditions as for the samples (Saá,1995). ß-glucosidase activity was determined as described for acid phosphomonoesterase activity except that the substrate was 25 mM *p*-nitrophenyl-ß-glucopyranoside and the *p*-nitrophenol released was extracted with 0.1 M THAM-NaOH at pH 12 (Eivazi and Tabatabai, 1988). Arylsulphatase activity was determined following the method described by Tabatabai and Bremner (1970), but using 2 M $CaCl_2$ and 0.2 M NaOH instead of 0.5 M $CaCl_2$ and 0.5 M NaOH, and quantifying the enzymatic activity by reference to calibration curves corresponding to *p*-nitrophenol standards incubated with each soil under the same conditions as for the samples (Saá,1995). For the latter four enzymes, the enzymatic activity was expressed in µmol *p*-nitrophenol g^{-1} h^{-1}. For each soil sample, all determinations were made in triplicate and the average values were expressed on an oven-dried soil basis.

Monitoring of plant survival, growth and ionome

The maximum shoot height of trees was measured 0.5, 1, 2 and 3 years after incorporation of the compost. Tree mortality was also recorded and dead trees were replaced during the first year. To monitor the plant nutritive status and shoot metal accumulation, sub-samples of the plant aerial biomass (shoots in the case of *A. capillaris*, and both stems and leaves in the woody species) were collected at each sampling time. Plant material was carefully washed with pressurised tap water followed by deionised water, oven-dried at 45°C and ground. Dried plant

material (approximately 0.1 g) were digested in a 2:1 concentrated HNO_3:HCl mixture on a hot plate at 120 °C, and the concentrations of P, K, Ca, Mg, Fe, Cd, Cu, Mn, Ni, Pb and Zn were measured by ICP-OES.

For *A. capillaris*, the total biomass produced in each sub-plot was harvested after two and three years (June 2013 and 2014, respectively). For tree species, biomass production was determined in each sub-plot after four years of growth (harvested in March 2015). Biomass was expressed as tonnes shoot dry weight (DW) yield per hectare.

Statistical analyses

At each sampling period, the effects of compost amendment and plant cover on soil physico- and bio-chemical properties, and plant growth and ionome, was assessed using a one-way analyses of variance (ANOVA) followed by a multiple comparison of means using the post-hoc Tukey-test for independent means. When necessary, data were transformed to meet the requirements of ANOVA. The statistical analysis was performed using IBM SPSS Statistics v22 for Windows.

8.3. Results

Effect of compost addition on soil physico- and bio-chemical properties

Table 8.1 lists the mean values (and range) for the main soil properties of the non-amended mine tailings. The tailings soil present a highly acidic pH and low fertility (reflected through the low total C and N content, and available P). The effective CEC was low and the H^+ and Al^{3+} cations were dominant. Trace element concentrations were highly variable across the tailings but, in general, elevated concentrations of Al, Cr, Cu, Fe, Mn or Ni were found (Table 8.1). Total Al and Fe concentrations fell within the same interval, with values varying from 33.4 to 58.9 g Al kg^{-1} and 81 to 137 g Fe kg^{-1}. Of the remaining TE, the principal metals were Cu and Mn: the mean total concentrations were 596 mg Cu kg^{-1} and 823 mg Mn kg^{-1}. Total concentrations of Cr and Ni ranged from 68 to 140 mg Cr kg^{-1} and from 27 to 181 mg Ni kg^{-1}. The highest values of available metals were found for Cu and Mn. The mean $NaNO_3$-extractable Cu concentration was 61 mg kg^{-1} but values oscillated between 7 and 219 mg kg^{-1} across the experimental plot. The mean $NaNO_3$-extractable concentration of Mn was 823 mg kg^{-1} (and ranged from 650 to 1142 mg kg^{-1}).

Table 8.1. Physicochemical properties of the mine tailings and compost

	Mine tailings soil		Compost
	Mean	**Range**	**Mean (± SE)**
pH_{H2O}	3.06	2.70 – 3.62	6.18 ± 0.04
pH_{KCl}	2.90	2.30 – 3.42	6.15 ± 0.05
EC (mS cm^{-1})	1.85	0.34 – 3.97	-
%C	0.60	0.10 – 1.08	15.4 ± 0.4
%N	0.10	0.08 – 0.13	1.4 ± 0.0
P olsen (mg kg^{-1})	2.5	<0.2 – 4.4	425.3 ± 33.0
CEC ($cmol_c$ kg^{-1})	21.4	7.9 – 49.3	57.6 ± 4.5
H^+	9.4	3.5 – 23.7	< loq
Al^{3+}	8.0	3.8 – 18.9	< loq
Ca^{2+}	2.4	0.3 – 8.1	40.4 ± 1.2
K^+	< loq		4.4 ± 0.1
Mg^{2+}	1.5	0.1 – 6.6	10.3 ± 0.5
Na^+	< loq		2.5 ± 0.1
Pseudo-total element concentrations			
g kg^{-1}			
P	0.7	0.5 – 1.0	11.8 ± 0.3
K	3.6	1.9 – 5.3	5.6 ± 0.2
Al	42.5	33.4 – 58.9	31.5 ± 0.4
Ca	5.9	2.9 – 12.8	30.1 ± 0.9
Mg	9.5	7.4 – 12.2	6.8 ± 0.2
Fe	105.0	81 – 137	40 ± 7
mg kg^{-1}			
Mn	823.0	650 – 1142	861 ± 20
Cu	596.0	308 – 1155	529 ± 84
Zn	123.0	95 – 366	530 ± 17
Cr	96.0	68 – 140	65.0 ± 1.6
Ni	43.0	27 – 181	59.2 ± 8.5
Cd	1.6	0.8 – 2.3	1.7 ± 0.2
Co	12.2	2.2 – 29.4	12.8 ± 0.5
$NaNO_3$-extractable metal concentrations (mg kg^{-1})			
Mn	63.0	14 – 289	-
Cu	61.0	7 – 219	-
Zn	21.5	2.3 – 91.7	-
Cr	<loq		-
Ni	9.0	0.7 – 40.7	-
Cd	0.12	0.01 – 0.60	-
Co	7.0	0.8 – 23.5	-

loq, limit of quantification: 0.01 $cmol_c$ kg^{-1} Al^{3+}, K^+; 0.04 $cmol_c$ kg^{-1} Na^+; 0.013 mg Cr kg^{-1}

Table 8.2 gives an overview of the changes induced in general soil properties over time after amendment with compost (independent of plant cover). One year after compost addition, soil pH was significantly increased, and continued to increase over the following two years ($p < 0.05$). A progressive increase in total C and N content was also observed through time, reaching maximal values after 2 years. These increments were maintained at year 3: the total C and N content at this time was 7.90% and 0.65% (representing an increase of 12- and 6-fold compared to non-amended soil, respectively). Compost addition also induced significant changes in the effective CEC. Soil CEC increased over the first year, reaching maximal values at this time of 33.2 $cmol_c\ kg^{-1}$.

Thereafter, CEC progressively dropped and after 3 years similar values to those determined in the non-amended tailings were obtained. However, before amending the tailings the CEC was dominated by the acidic cations H^+ and Al^{3+} (with a base saturation of <20%), while after three years the mean base saturation was >90% (predominantly dominated by Ca^{2+}; Table 8.2). Exchangeable Ca increased from 2.3 $cmol_c\ kg^{-1}$ in non-amended tailings soil to 29.3 $cmol_c\ kg^{-1}$ in year 1, thereafter decreasing to 14.8 $cmol_c\ kg^{-1}$ in year 3. Similar trends were observed in exchangeable Mg^{2+} and K^+, although these were less pronounced.

Table 8.2. Evolution of soil physicochemical properties in the mine tailings soil after amendment with compost (considering the whole experimental plot). Different letters indicate significant differences with time ($p < 0.05$).

	T =0	T = 1 year	T =2 years	T = 3 years
pH $_{H2O}$	3.18 ± *0.02*c	5.37 ± *0.15*b	6.22 ± *0.11*a	6.48 ± *0.11*a
pH $_{KCl}$	3.06 ± *0.02*c	5.16 ± *0.15*b	5.71 ± *0.10*a	6.04 ± *0.09*a
Cu concentration (mg kg^{-1})				
Pseudo-total	523 ± *21*a	467 ± *22*a	529 ± *41*a	481 ± *29*a
$NaNO_3$-extractable	33.0 ± *3.1*a	1.2 ± *0.4*b	0.3 ± *0.1*b	0.4 ± *0.1*b
CEC ($cmol_c\ kg^{-1}$)	19.5 ± *3.5*b	33.2 ± *3.9*a	28.5 ± *1.6*a	17.4 ± *0.8*b
H^+	7.96 ± *1.22*a	0.16 ± *0.11*b	< loq	< loq
Al^{3+}	7.78 ± *1.28*a	0.44 ± *0.28*b	0.20 ± *0.09*b	0.18 ± *0.09*b
Ca^{2+}	2.3 ± *0.6*c	29.3 ± *3.7*a	24.8 ± *1.4*ab	14.8 ± *0.7*b
K^+	< loq	0.85 ± *0.13*a	*0.95* ± *0.06*a	0.64± *0.06*a
Mg^{2+}	1.45 ± *0.50*b	2.43 ± *0.42*a	2.68 ± *0.15*a	1.73 ± *0.09*ab
Polsen (mg kg^{-1})	2.1 ± *0.3*c	243.0 ± *29.8*a	194.3 ± *10.1*ab	133.3 ± *10.6*b
%C	0.63 ± *0.08*b	6.67 ± *0.72*a	8.14 ± *0.72*a	7.91 ± *0.94*a
%N	0.11 ± *0.00*c	0.45 ± *0.07*b	0.70 ± *0.06*a	0.65 ± *0.05*a

loq, limit of quantification: 0.01 $cmol_c\ K\ kg^{-1}$

Available P (P olsen) also showed a pronounced increase after compost addition: $NaHCO_3$-extractable P concentrations peaked at year 1 (representing an increase of approximately 100-fold compared to non-amended tailings soil; $p < 0.05$) and then progressively dropped during the following two years but never reached levels of non-amended tailings soil. Compost addition did not significantly alter the pseudo-total concentration of Cu (Table 8.2). However, a significant increase in the total concentration of Mn, Pb and Zn was observed: their concentrations (in mg kg^{-1}) increased from 882 to 1119 for Mn, 16.8 to 46.4 for Pb, and 125.0 to 311.5 for Zn. Soil amendment had a strong effect on TE availability (estimated with the $NaNO_3$-extractable concentration). The mean $NaNO_3$-extractable Cu concentration in the mine tailings soil before compost addition was 61 mg kg^{-1} and decreased to 2.9 mg kg^{-1} after one year and to 0.5 mg kg^{-1} after three years ($p < 0.05$). $NaNO_3$-extractable concentrations of TE such as Al, Cd, Co, Cr, Ni and Zn were below the quantification limit (0.025 mg kg^{-1} for Cd, Mn and Ni, 0.0125 mg kg^{-1} for Co and Cr and 0.125 mg kg^{-1} for Zn) after three years, and the mean Mn concentration in this labile pool was 1.4 mg kg^{-1}.

The lowest soil enzyme activities were always found in the non-amended tailings soil, and the compost addition induced a significant increase in all assayed enzymes (Table 8.3). In non-amended soil the activity of the oxido-reductase enzymes was null, while one year after compost amendment mean values of catalase and dehydrogenase activities of 1.17 mmol H_2O_2 consumed g^{-1} h^{-1} and 0.28 µmol INTF g^{-1} h^{-1} were recorded. For the hydrolase enzymes, enzymes activities were increased by 4- to 23-fold (depending on the enzyme) after one year. The

Table 8.3. Evolution of soil enzyme activities in the mine tailings soil after amendment with compost (considering the whole experimental plot). Different letters indicate significant differences with time ($p < 0.05$).

	T =0	T = 1 year	T =2 years	T = 3 years
Catalase (mmol H_2O_2 consumed g^{-1} h^{-1})	0.00 ± *0.00*c	1.27 ± *0.14*ab	0.84 ± *0.12*b	1.56 ± *0.11*a
Dehydrogenase (µmol INTF g^{-1} h^{-1})	0.01 ± *0.00*c	0.28 ± *0.02*a	0.18 ± *0.02*b	0.19 ± *0.02*b
Invertase (µmol glucose g^{-1} h^{-1})	0.50 ± *0.02*c	4.64 ± *0.55*ab	3.84 ± *0.36*b	6.63 ± *0.63*a
ß-glucosidase (µmol PNP g^{-1} h^{-1})	0.12 ± *0.02*c	0.43 ± *0.05*c	0.74 ± *0.06*b	1.06 ± *0.09*a
Urease (µmol NH_3 g^{-1} h^{-1})	0.16 ± *0.03*c	3.74 ± *0.31*ab	2.59 ± *0.22*b	4.83 ± *0.38*a
Acid phosphomonoesterase (µmol PNP g^{-1} h^{-1})	0.41 ± *0.04*c	1.57 ± *0.12*ab	1.70 ± *0.11*a	1.23 ± *0.06*b
Alkaline phosphomonoesterase (µmol PNP g^{-1} h^{-1})	0.21 ± *0.01*b	1.16 ± *0.13*a	0.70 ± *0.11*ab	0.85 ± *0.1*ab
Arylsulphatase (µmol PNP g^{-1} h^{-1})	0.00 ± *0.00*c	0.08 ± *0.01*ab	0.06 ± *0.01b*	0.12 ± *0.01*a

most pronounced increases were observed in urease (mean values increased from 0.16 µmol NH_3 g^{-1} h^{-1} to 3.74 µmol NH_3 g^{-1} h^{-1}) and arylsulphatase (no activity was observed in non-amended tailings and after one year a mean value of 0.08 µmol PNP g^{-1} h^{-1} was obtained). In general, these significant increments in activities were maintained over the experimental period in all enzymes except dehydrogenase, while a further significant increase in the activities of invertase and β-glucosidase was observed between 2 and 3 years (Table 8.3).

Evolution in plant growth and shoot ionome

The maximum height (per individual) of both *Salix* spp. and *P. nigra* was recorded after the first growth season (6 months), and thereafter, on a yearly basis until harvesting (after 4 years; Figure 8.2). A large variability in plant height was observed between individuals of the same species across the three replicate subplots. After the first growth season the mean maximum height recorded was 106 cm for *S. viminalis*, 80 cm for *S. caprea* and 96 cm for *P. nigra*. Plant height did not change significantly between 6 months and 1 year, but individuals of all three

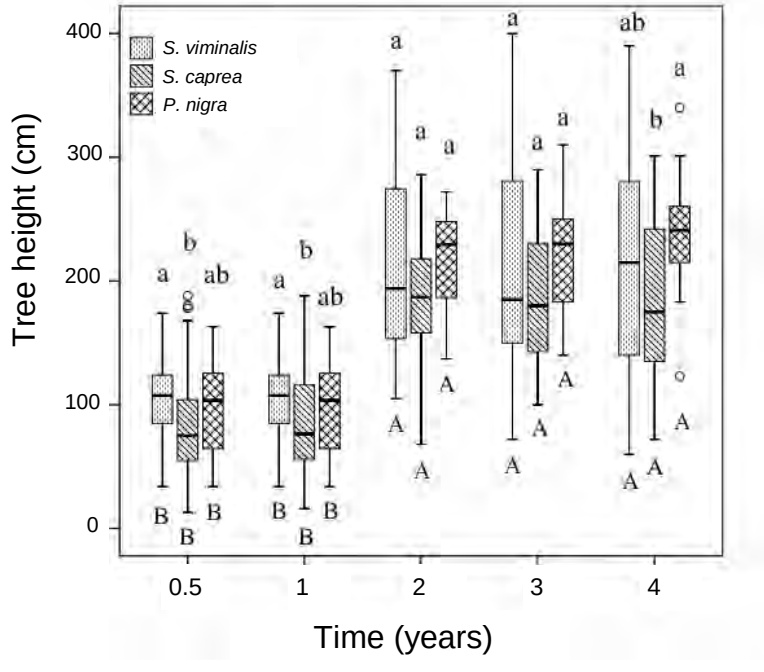


Figure 8.2. Maximum shoot height (cm) of *Salix viminalis*, *Salix caprea* and *Populus nigra*. Boxes represent the median (vertical line) and 25 and 75% quartiles. Whiskers represent the 90th and 10th percentile. Outliers are represented by unfilled circles. Different capital letters denote significant differences between years within each plant species and different lower case letters indicate significant differences between plant species within the same year ($p < 0.05$).

species were significantly taller after 2 years ($p < 0.05$). Mean maximal height was 217 cm, 184 cm and 217 cm in *S. viminalis*, *S. caprea* and *P. nigra* after two years (representing an increase of 2.0-, 2.3- and 2.3-fold, respectively). No significant change in plant height was recorded between years 2 and 4 of the experiment. During the first year, plant height was significantly lower in *S. caprea*, and this tended to be the case throughout the four years of the experiment (Figure 8.2). At harvest (after four years of growth), the mean height for *S. viminalis* was 219 cm (with plants reaching up to 390 cm), 189 cm for *S. caprea* (with a maximum of 340 cm), and 240 cm for *P. nigra* (maximum of 300 cm). After 4 years, plant mortality was generally higher for *Salix* spp. than *P. nigra*. Survival of *P. nigra* was 67-75% (of initial planted trees), while survival of both *Salix* spp. was similar but highly variable between sub-plots: ranging from 35 to 96% in the case of *S. viminalis* and from 35 to 87% in the case of *S. caprea*. Consequently, shoot DW yield obtained after 4 years of growth was highly variable between replicate sub-plots and significant differences between the three species were not observed. The mean shoot DW yield showed a greater variability in *Salix* spp. (ranging from 3.7 ±3.0 t ha^{-1} in *S. caprea* to 4.3 ±2.3 t ha^{-1} in *S. viminalis*). In contrast, shoot yields were more uniform between sub-plots of *P. nigra*, where a mean biomass production of 4.3 ±0.7 t ha^{-1} was obtained. *Agrostis capillaris* presented a good establishment and coverage within the first year of seeding. Shoot DW yield reached 2.2 t ha^{-1} at the first cutting (after 2 years), and this increased to 4.6 t ha^{-1} at the second cutting (after 3 years).

Leaf and stem ionome of the three woody species are presented in Tables 8.4a and 8.4b, respectively. Concentrations of Ca, K, Co, Fe and Mn were significantly higher in leaves than in stems ($p < 0.05$) while for Mg, P, Cu and Zn no differences between tissues were observed ($p > 0.05$). Element accumulation depended on the plant species, plant tissue and year. Leaf Ca concentrations (in g kg^{-1}) of *S. viminalis*, *S. caprea* and *P. nigra* were in the range of 13.9-16.4, 8.9-22.6 and 9.9-23.4 (Table 8.4a). The lowest concentrations were generally observed after three years of growth (albeit not always statistically significant). Leaf K, Mg and P concentrations were maximal at year 1 (Table 8.4a) and were reduced by 1.5- to 2-fold in the second and third year (with few differences between species). No significant differences were observed with time in leaf concentrations of micronutrients such as Co, Fe and Mn, while leaf Zn concentrations were lowest in the third year of growth ($p < 0.05$). In contrast, leaf Cu concentrations were generally highest after three years, and ranged from 13.4 to 19.6 mg

Table 8.4a. Macro- and micro-nutrient concentrations in leaves of woody tree species (mean concentration ± SE) at the three sampling times. Different capital letters denote significant differences between years within each plant species and different lower case letters indicate significant differences between plant species within the same year ($p < 0.05$).

		Ca	K	Mg	P	Co	Cu	Fe	Mn	Zn
		g kg^{-1}				mg kg^{-1}				
2012	***S. viminalis***	15.6 ± *1.0*Ab	25.6 ± *1.3*Aa	5.6 ± *0.3*Aa	4.4 ± *0.2*Aa	3.0 ± *0.3*Aa	11.3 ± *0.5*Ab	136.9 ± *1.4*Aa	151.5 ± *28.0*Aa	191.7 ± *25.8*Ab
	S. caprea	15.0 ± *0.3*Bb	19.6 ± *0.7*Ab	3.7 ± *0.2*Ab	4.5 ± *0.1*Aa	2.9 ± *0.8*Aa	13.8 ± *0.9*ABa	171.6 ± *20.4*Aa	150.3 ± *39.2*Aa	285.3 ± *4.2*ABa
	P. nigra	23.4 ± *1.3*Aa	20.0 ± *0.7*Ab	7.1 ± *0.6*Aa	5.2 ± *0.6*Aa	3.3 ± *0.3*Aa	9.2 ± *0.3*Bb	136.5 ± *7.9*Aa	247.4 ± *39.8*Aa	180.5 ± *22.5*Ab
2013	***S. viminalis***	16.4 ± *0.4*Ab	16.0 ± *1.1*Ba	2.6 ± *0.0*Bb	2.9 ± *0.4*Ba	1.9 ± *0.7*ABa	7.2 ± *1.1*Ba	145.9 ± *22.0Aa*	90.8 ± *31.8*Aa	151.7 ± *23.2*ABb
	S. caprea	22.6 ± *2.2*Aa	12.0 ± *1.0*Ba	2.1 ± *0.2*Bb	3.0 ± *0.1*Ba	1.9 ± *0.6*Aa	7.8 ± *1.4*Ba	123.4 ± *9.2*Ba	167.8 ± *35.1*Aa	430.8 ± *73.1*ABa
	P. nigra	14.3 ± *1.3*Bb	13.2 ± *0.4*Ba	4.1 ± *0.5*Ba	2.6 ± *0.3*Ba	3.0 ± *0.1*Aa	8.7 ± *0.7*Ba	124.2 ± *16.5*Aa	121.7 ± *6.6*Ba	148.4 ± *12.3*ABb
2014	***S. viminalis***	13.9 ± *1.1*Aa	15.2 ± *0.3*Ba	2.5 ± *0.4*Ba	3.2 ± *0.4*ABa	0.8 ± *0.2*Bb	13.4 ± *0.7*Aa	131.8 ± *19.7*Aa	82.1 ± *17.2*Ab	88.0 ± *8.3*Bb
	S. caprea	8.9 ± *0.3*Cb	14.6 ± *0.3*Ba	2.2 ± *0.1*Ba	4.3 ± *0.1*Aa	1.4 ± *0.1*Aab	19.6 ± *3.1*Aa	187.3 ± *11.3*Aa	174.1 ± *12.4*Aa	191.3 ± *31.3*Ba
	P. nigra	9.9 ± *0.1*Bb	16.0 ± *0.8*Ba	2.4 ± *0.2*Ba	3.3 ± *0.2*Ba	1.6 ± *0.1*Ba	13.6 ± *0.0*Aa	153.8 ± *5.6*Aa	142.5 ± *18.5*ABab	105.4 ± *0.2*Bb

Table 8.4b. Macro- and micro-nutrient concentrations in stems of woody tree species (mean concentration ± SE) at the three sampling times. Different capital letters denote significant differences between years within each plant species and different lower case letters indicate significant differences between plant species within the same year ($p < 0.05$).

		Ca	K	Mg	P	Co	Cu	Fe	Mn	Zn
		g kg^{-1}				mg kg^{-1}				
	S. viminalis	8.8 ± *0.5*Ab	26.6 ± *1.5*Aa	2.6 ± *0.3*Ab	3.5 ± *0.2*Ab	0.9 ± *0.1*Aa	13.8 ± *0.9*Aa	115.8 ± *12.3*Aa	78.3 ± *17.0*Aa	128.6 ± *8.5*Ab
2012	***S. caprea***	9.5 ± *0.2*ABb	19.0 ± *0.7*Ab	2.5 ± *0.1*Ab	3.6 ± *0.1*Ab	*1.0* ± *0.4*Aa	14.2 ± *3.5*Aa	118.1 ± *24.2*Aa	66.5 ± *13.3*Aa	188.6 ± *8.9*Aa
	P. nigra	17.4 ± *0.3*Aa	26.2 ± *0.6*Aa	5.2 ± *0.3*Aa	5.1 ± *0.5*Aa	1.2 ± *0.1*Aa	10.3 ± *0.3*Aa	94.6 ± *0.2*Aa	98.4 ± *3.6*Aa	101.3 ± *0.7*Ab
	S. viminalis	7.8 ± *0.4*Ab	9.8 ± *0.7*Bb	0.9 ± *0.1*Bb	2.2 ± *0.3*Ba	0.6 ± *0.2*Aa	6.0 ± *0.8*Bb	89.2 ± *30.5*Aa	41.2 ± *11.6*Aab	75.2 ± *9.5*Ab
2013	***S. caprea***	12.3 ± *0.6*Aa	9.3 ± *1.0*Bb	1.2 ± *0.0*Bab	2.2 ± *0.1*Ba	0.6 ± *0.2*Aa	9.3 ± *0.6*ABab	91.4 ± *28.7*Aa	93.4 ± *25.6*Aa	196.7 ± *25.1*Aa
	P. nigra	7.9 ± *0.7*Bb	15.8 ± *0.1*Ba	1.5 ± *0.1*Ba	3.1 ± *0.1*Ba	0.6 ± *0.1*Ba	13.7 ± *1.1*Aa	62.6 ± *11.3*Aa	30.4 ± *1.4*Bb	60.5 ± *0.2*Bb
	S. viminalis	8.0 ± *0.7*Aa	8.4 ± *1.1*Ba	1.2 ± *0.1*Ba	1.8 ± *0.2*Ba	0.4 ± *0.1*Aa	9.5 ± *0.7*ABb	87.3 ± *14.9*Aa	63.5 ± *9.6*Aa	81.6 ± *5.6*Aa
2014	***S. caprea***	8.8 ± *1.6*ABa	10.7 ± *0.9*Ba	*1.0* ± *0.1*Ba	1.8 ± *0.1*Ca	0.3 ± *0.1*Aa	14.1 ± *0.5*ABa	92.8 ± *22.6*Aa	69.4 ± *27.7*Aa	138.7 ± *55.5*Aa
	P. nigra	7.7 ± *0.2*Ba	11.1 ± *0.1*Ca	*1.1* ± *0.0*BCa	2.1 ± *0.1*Ca	0.4 ± *0.1*BCa	14.6 ± *0.2*Aa	73.7 ± *1.3*Aa	38.0 ± *1.8*Ba	58.9 ± *2.5*Ba
	S. viminalis	9.9 ± *0.6*Aa	4.4 ± *0.4*Ca	0.8 ± *0.0*Ba	1.5 ± *0.1*Ba	0.6 ± *0.2*Aa	9.7 ± *1.1*Ba	73.3 ± *4.5*Aa	91.9 ± *11.5*Aa	102.5 ± *13.6*Aa
2015	***S. caprea***	7.1 ± *0.6*Bb	2.8 ± *0.2*Cb	0.5 ± *0.0*Cb	1.2 ± *0.1*Da	*0.1* ± *0.0*Aa	7.7 ± *0.6*Ba	124.8 ± *60.9*Aa	62.9 ± *3.2*Aab	133.0 ± *13.1*Aa
	P. nigra	6.4 ± *0.4*Bb	4.3 ± *0.3*Da	0.9 ± *0.0*Ca	1.6 ± *0.1*Ca	0.3 ± *0.0*Ca	9.0 ± *1.4*Aa	148.8 ± *38.4*Aa	39.4 ± *3.3*Bb	52.9 ± *3.5*Bb

kg^{-1} with no difference between species. Stem Ca concentrations (in g kg^{-1}) of *S. viminalis*, *S. caprea* and *P. nigra* were in the range of 7.8-9.9, 7.1-12.3 and 6.4-17.4 (Table 8.4b). There was no consistent trend in Ca accumulation in woody parts with time amongst the three tree species: maximal stem Ca concentrations were observed after one year of growth in *P. nigra* (thereafter significantly declining), two years in *S. caprea*, and four years in *S. viminalis*. Stem K, Mg, and P concentrations showed significant and progressive reductions with time in all three species. At harvest (after 4 years), stem concentrations of these three elements ranged from 2.8 to 4.4 g K kg^{-1}, 0.5 to 0.9 g Mg kg^{-1} and 1.2 to 1.6 g P kg^{-1}. Stem Cu concentrations oscillated in the range of 6.0-13.8 mg kg^{-1} in *S. viminalis*, 7.7-14.2 mg kg^{-1} in *S. caprea* and 9.0-14.6 mg kg^{-1} in *P. nigra*. In contrast to what was observed in leaves, stem Cu concentrations decreased significantly with time (with the exception of *P. nigra*). Stem concentrations of Fe did not vary with time or between species, while Co concentrations did not vary between species but decreased with time in the case of *P. nigra*. Similar trends were observed in stem Mn and Zn concentrations (Table 8.4b).

Table 8.5 shows the changes in shoot ionome of *A. capillaris* over time. Shoot Ca concentrations showed a slight but significant reduction over time (from 3.5 g kg^{-1} after 1 year to 3.2 g kg^{-1} after 3 years). Shoot K, Mg and P concentrations varied according to the year, with the lowest concentrations generally being found after 2 years of cultivation. After 3 years, the mean shoot K, Mg and P concentration was 14.4 g kg^{-1}, 1.1 g kg^{-1} and 2.6 g kg^{-1}, respectively. Shoot concentra-

Table 8.5. Macro- and micro-nutrient concentrations in *Agrostis capillaris* shoot (mean concentration ± standard error) at the three sampling times. Different capital letters denote significant differences between different years ($p < 0.05$).

	Ca	K	Mg	P	
			g kg^{-1}		
2012	3.5 ± *0.1*A	16.1 ± *0.2*A	1. 3 ± *0.0*A	2.5 ± *0.1*A	
2013	3.1 ± *0.1*B	3.7 ± *0.1*B	0.9 ± *0.0*C	1.3 ± *0.0*B	
2014	3.2 ± *0.0*B	14.4 ± *0.4*A	1.1 ± *0.0*B	2.6 ± *0.1*A	
	Co	**Cu**	**Fe**	**Mn**	**Zn**
			mg kg^{-1}		
2012	0.31 ± *0.20*B	5.2 ± *0.3*B	72.3 ± *2.8*C	145.5 ± *27.0*B	48.6 ± *2.6*AB
2013	2.81 ± *0.02*A	12.8 ± *0.3*A	793.7 ± *38.7*A	256.7 ± *6.7*A	55.6 ± *1.8*A
2014	0.25 ± *0.04*B	12.0 ± *0.6*A	243.1 ± *28.5*B	97.7 ± *7.3*C	41.8 ± *1.8*B

tions of micronutrients, such as Co, Fe, Mn and Zn, also varied from year to year. However, shoot Cu concentrations increased with time: its values were 5.2 mg kg^{-1} after one year and peaked to 12.0-12.8 mg kg^{-1} in years 2 and 3 (Table 8.5).

Effects of plant species on soil physico- and bio-chemical properties

The main changes observed in soil physicochemical properties were the result of the compost addition itself rather than due to plant growth and activity. However, some significant effects of plant cover on soil general properties were observed. Figure 8.3 shows the effects of plant species on soil pH in the compost-amended plots over time. In general, soil pH in compost-amended tailings was higher under plant cover than in unplanted plots. After one year of growth this plant-induced effect was only significant in the case of *S. viminalis*: soil pH increased from 4.8 to 6.2 ($p < 0.05$). However, in both subsequent years this increase was significant for all three woody species and also for *A. capillaris* ($p < 0.05$). Plant-induced effects were also observed in CEC in the final two years of monitoring (Figure 8.4). Both woody trees and the grassy species significantly increased soil CEC after two years of cultivation compared to unplanted soil: CEC

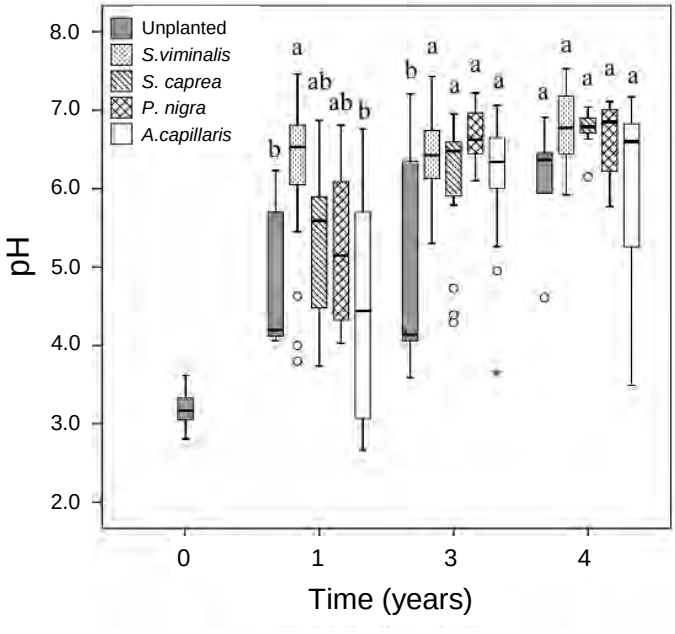


Figure 8.3. Effects of plant species (*Salix viminalis, S. caprea, Populus nigra* and *Agrostis capillaris*) on soil pH. Boxes represent the median (vertical line) and 25 and 75% quartiles. Whiskers represent the 90th and 10th percentile. Outliers are represented by unfilled circles. Different capital letters denote significant differences between years within each plant species and different lower case letters indicate significant differences between plant species within the same year ($p < 0.05$).

increased from 12.1 $cmol_c$ kg^{-1} in unplanted sub-plots to 27.6-29.5 $cmol_c$ kg^{-1} under tree cover and to a maximum of 35.8 under *A. capillaris*. In contrast, this effect was less clear after three years, as a higher CEC was only observed in soils planted with *A. capillaris*. The presence of a plant cover also tended to increase available P (P olsen) compared to unplanted plots ($NaHCO_3$-extractable P concentrations increased from 118 mg kg^{-1} in compost-amended unplanted soil to 206 mg kg^{-1} in soils cultivated with *P. nigra*). However, this effect was not statistically

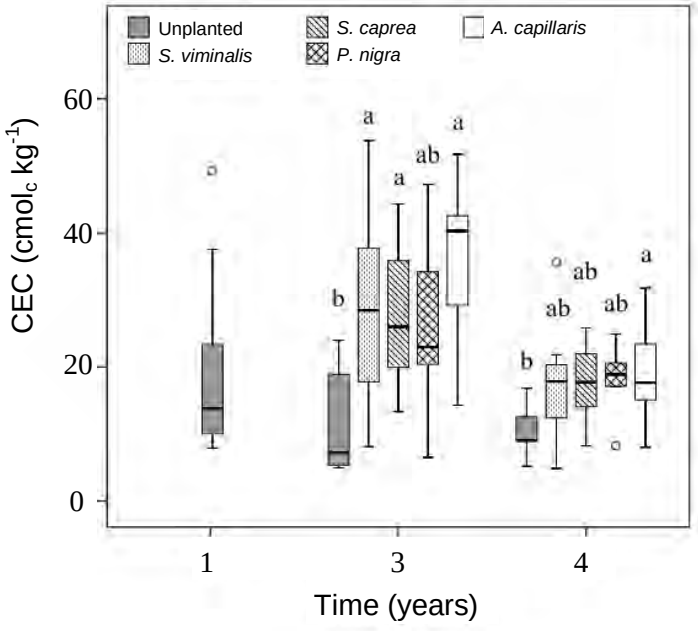


Figure 8.4. Effect of plant species (*Salix viminalis*, *S. caprea*, *Populus nigra* and *Agrosis capillaris*) on soil cation exchange capacity (CEC). Boxes represent the median (vertical line) and 25 and 75% quartiles. Whiskers represent the 90th and 10th percentile. Outliers are represented by unfilled circles. Different capital letters denote significant differences between years within each plant species and different lower case letters indicate significant differences between plant species within the same year ($p < 0.05$).

significant (data not shown). In general, the strong reduction observed in available Cu after compost amendment was not significantly influenced by plant cover (Figure 8.5). A difference in this parameter between planted and unplanted plots was only observed at one sampling point (2 years after amendment addition): $NaNO_3$-extractable Cu concentrations were significantly higher in unplanted soil (mean concentration of 2.27 mg kg^{-1}) than under plant cover (where a mean concentration of 0.16 – 0.26 mg kg^{-1} was determined). After three years no effect of plant cover was found, and available Cu was <1 mg kg^{-1} in all soils.

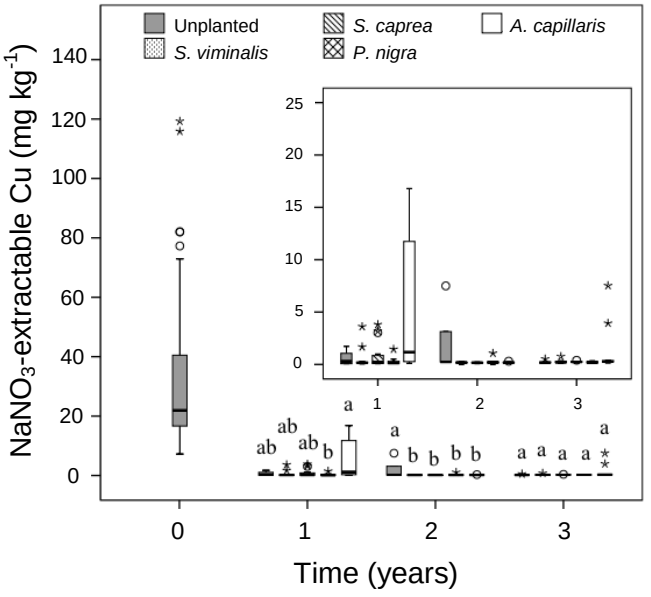


Figure 8.5. Effect of plant species (*Salix viminalis, S. caprea, Populus nigra* and *Agrostis capillaris*) on soil $NaNO_3$-extractable Cu concentrations. Boxes represent the median (vertical line) and 25 and 75% quartiles. Whiskers represent the 90th and 10th percentile. Outliers are represented by unfilled circles. Different capital letters denote significant differences between years within each plant species and different lower case letters indicate significant differences between plant species within the same year ($p < 0.05$).

Plant cover had a significant effect on biochemical parameters. Figure 8.6 presents the soil enzyme activities for non-amended mine tailings at the beginning of the experiment, and the evolution in activities over time in unplanted soil, and soils cultivated with either *S. viminalis* or *A. capillaris*. The mean values of enzyme activities were always higher in planted soils than in unplanted soils, and this was the case for all sampling time points and all assayed enzymes (Figure 8.6). In general, enzyme activities in planted soils did not progressively increase over time, although urease, invertase, β-glucosidase and arylsulphatase activities were significantly higher after 3 years than at either 1 or 2 years. However, enzyme activities were influenced by plant species, and the mean values in soils under *S. viminalis* were generally higher than those recorded under *A. capillaris* (albeit not always statistically significant). For example, after 2 years the activities of catalase and dehydrogenase were 1.5- and 1.6-fold higher in soil under *S. viminalis* than *A. capillaris* ($p < 0.05$). A similar effect was observed in P cycle enzymes: the activity of acid and alkaline phosphomonoesterase (in µmol PNP g^{-1} h^{-1}) was 1.65 and 0.44 in soil under *A. capillaris* but increased to 2.05 and 1.16 in soil under *S. viminalis* (not significant in the case of acid phosphomonoesterase).

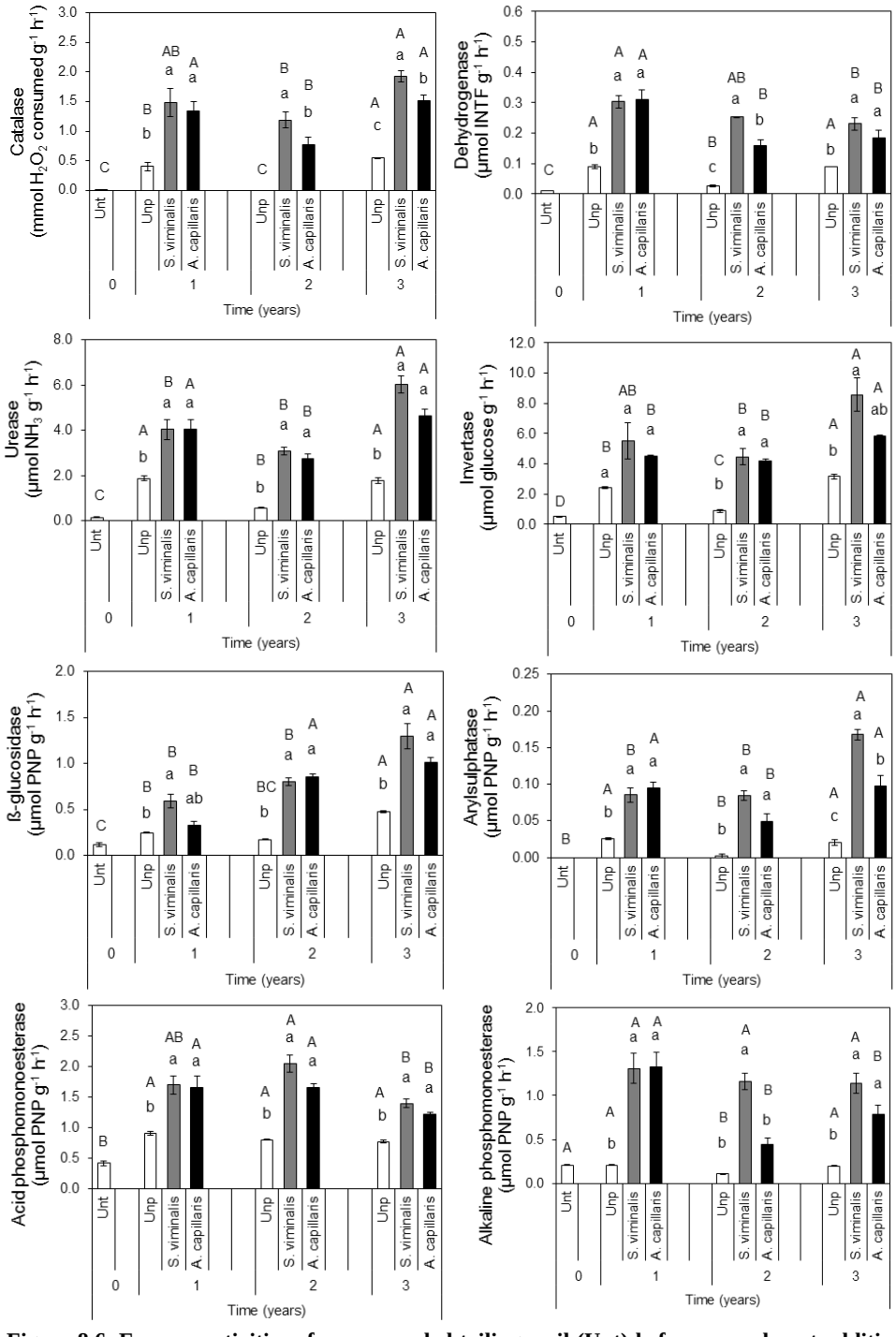


Figure 8.6. Enzyme activities of non-amended tailings soil (Unt) before amendment addition, unplanted compost-amended soil (Unp), and compost-amended soil with a *Salix viminalis* or *Agrostis capillaris* cover. Lower case letters indicate significant differences between the treatments at each sampling time ($p < 0.05$). A capital letter shows a significant difference within the same treatment over time ($p < 0.05$).

Finally, arylsulphatase (of the S cycle) activity was also 1.6-fold higher in the *S. viminalis* soil (but this did not reach significance). After 3 years higher activities under *S. viminalis* were evident in all the enzymes assayed (although statistical significance was only reached for catalase and arylsulphatase; Figure 8.6).

8.4. Discussion

The Touro mine tailings present an extremely inhospitable environment for plant growth and are generally bare of vegetation when no remediation measures are taken. The uncontrolled oxidation of sulphides generates hyperacidic and hyperoxidising soils and waters, and the release of excessive concentrations of soluble metal sulphates. Moreover, the tailings soil is deficient in nutrients such as N, P and K, and scarcely contains OM. The selected experimental area was completely barren of vegetation before initiating the experiment, and microbial activity (estimated through catalase and dehydrogenase activities) was practically non-existent. The compost addition had a strong effect on all physicochemical properties, trace metal availability and biological activity. The improved soil properties permitted plant establishment and growth of both woody and grass species during the four years of the experiment.

Before amendment incorporation the tailings presented pH values of below 4 (and often less than 3). At these pH levels plants are exposed to toxic levels of soluble Al and H (exchangeable Al and H was up to 9.6 $cmol_c\ kg^{-1}$ and 15.3 $cmol_c\ kg^{-1}$, respectively), as well as other potentially phytotoxic elements that are present, such as Cu. Amending with compost had a strong and significant effect on soil pH, which progressively increased with time and reached values of around 6.5 after three years. Corresponding reductions were observed in exchangeable Al and H, and the soil cation exchange complex shifted from dominance by these acidic cations to a predominantly Ca-based CEC. Compost addition also had a beneficial effect in terms of reducing the availability of potentially toxic trace metals, and particularly on available Cu (estimated through $NaNO_3$-extractable concentrations). Initial $NaNO_3$-extractable concentrations of Cu reached values of up to 269 $mg\ kg^{-1}$ in the non-amended tailings soil. Although Cu is an essential element to plants (and is a cofactor of several enzymes), when it occurs in excess it is harmful to most plants and has the capacity to initiate oxidative damage and interfere with important cellular processes such as photosynthesis, pigment synthesis, plasma membrane permeability and other metabolic mechanisms which strongly limit the plant development (Van Assche and Clijsters, 1990). Organic wastes can

reduce the solubility and availability of metals (including Cu) in contaminated soils, mainly due to their alkaline character and the complexing capacity of OM (Mench *et al.,* 2003; de la Fuente *et al.,* 2011; Alvarenga *et al.,* 2014). Composted organic residues present a high proportion of humified OM which decreases metal availability through adsorption and the formation of stable organo-metal complexes (Clemente and Bernal, 2006). In addition, cationic metals bind to exchange sites on the OM surface, which reduces their mobility, avoiding metal leaching from soils (Soler-Rovira *et al.,* 2010). Soil pH was the most important chemical property governing Cu solubility and bioavailability in metal-contaminated soils amended with composted biosolids (Soler-Rovira *et al.,* 2010), but soil OM and the humic acid fraction, particularly binding sites formed by N-, S-, and O-containing acidic functional moieties in humic acids, were pivotal in Cu mobility. Nonetheless, the medium- and long-term effectiveness of these organic amendments will depend on the evolution of such organo-metallic compounds, since their solubility can change over time as a result of various factors (i.e. oxidation of metal sulphides, reduction of Fe and Mn oxides, and mineralisation of organo-metal complexes) (Madejón *et al.,* 2010; Pérez-de-Mora *et al.,* 2011). Here, the maximal reductions in Cu availability in planted compost-amended plots were found in year 1 and were maintained over the full three years.

Similarly, other field-based trials in trace element-contaminated soils have shown the potential benefits of organic-based amendments. Pardo *et al.* (2013) successfully used olive-mill waste compost as a soil amendment to promote the growth of a native legume (*Bituminaria bituminosa*) in a mine-affected soil from a semi-arid area (SE Spain) contaminated with trace elements (As, Cd, Cu, Mn, Pb and Zn). Compost addition increased the total soil organic C content and maintained elevated available P concentrations throughout two years. In an earlier study in the same mining area, Clemente *et al.* (2012) found compost and pig slurry to increase soil nutrient and OM contents and stimulate biomass and ground cover of the halophytic shrub *Atriplex halimus*. However, in both these studies amendments were shown to have little effect on labile trace metal concentrations or their phytoavailability. In a semi-field experiment, Alvarenga *et al.* (2014) found both mixed municipal solid waste compost and green waste-derived compost to be effective immobilising agents in aided phytostabilisation of a highly acidic soil contaminated with trace elements (As, Cd, Cu, Mn, Pb and Zn) and planted with *Agrostis tenuis*. Both amendments had an equivalent capacity to raise soil OM and pH, and to decrease bioavailable Cu and Zn. Best amendments for restoring plant

growth and immobilising Cu in a Cu-contaminated soil from a wood preservation site were alkaline materials such as dolomitic limestone, composted poultry manure and pine bark, and their combination (Bes and Mench, 2008). Farrell and Jones (2010) evaluated the potential of a range of waste-derived composts (from municipal wastes and green wastes) of varying quality in the presence and absence of lime to alleviate trace metal toxicity in highly acidic heavily contaminated soil (As, Cu, Pb, Zn). Overall, amelioration of metal toxicity was enhanced by compost whilst lime had little effect and even exacerbated mobilisation of elements, such as As. All composts reduced metal concentrations in the soil solution, while raising soil pH and nutrient levels and were well suited to revegetation of contaminated sites. However, these authors highlighted the need to ensure correct pH management (pH 5–6) to optimise plant growth whilst minimising metal solubilisation, particularly at higher pH values.

Here, two vegetation covers were effectively established: a grass cover and a SRC system based on *Salix* spp. and *P. nigra*. A pronounced plant-induced effect on soil physicochemical properties and Cu availability was generally not observed, and the main factor influencing these parameters was the compost amendment itself rather than plant growth. Biomass production obtained in the *A. capillaris* plots was similar to that reported by Alvarenga *et al.* (2014) for *A. tenuis* (up to 3 t ha^{-1}) cultivated in pyrite mine soils amended with green waste-derived compost. The willows and poplars rapidly accumulated biomass, reaching their maximum height after 2 years. Heavily contaminated mine tailings are generally considered unsuitable for the cultivation of food or fodder crops but the production of energy crops, such as SRC of willow and poplar, offer a sustainable method for the phytomanagement of this type of land. SRC systems have been successfully implemented in other contaminated areas. Ruttens *et al.* (2011) established a large-scale experimental field site in the Campine region (Belgium) with diverse willow and poplar clones. Mean annual wood biomass production (with plant densities of 18000 cuttings ha^{-1}) ranged from 1.1 t ha yr^{-1} (with *P. trichocarpa* x *P. deltoides*) to 6.6 t ha-1 yr^{-1} (with *S. viminalis*) after the first growth period. Maxted *et al.* (2007) implemented SRC with different *Salix* clones in a sewage sludge disposal facility (UK) and obtained stem biomass ranging from 5.6 t ha^{-1} to 12.0 t ha^{-1}. The average biomass productivity in this study (3.4 to 4.3 t ha^{-1}) was closer to the lower range obtained at both these field sites, although in some sub-plots a stem biomass of up to 10 t ha^{-1} was achieved. During the four years, periods of high temperatures and low precipitation were recorded leading to

the death of many trees: in 2012, during May to September maximum temperatures of 32 to 35ºC, and a total precipitation of <30-40 L m^{-2} in July-August, were recorded; while in 2013, during the same periods maximum temperatures oscillated between 30 to 36ºC with a total precipitation of 30-40 L m^{-2}. Mortality in some sub-plots reached up to 65% and was most likely due to insufficient irrigation, a factor which needs careful consideration when implementing this kind of technique. Willows have high irrigation requirements for successful establishment and productivity, and under water stress conditions, they do not maintain the same level of biomass production (Kidd *et al.*, 2015).

The nutrient concentrations in both the woody trees and *A. capillaris* in the compost-amended soils were within the optimal range for plant growth (according to Marschner (1995)). Although some reductions were observed over time (to a lesser extent in *A. capillaris* than the trees) levels of nutrient deficiency were never reached. For successful phytostabilisation it is important to select metal tolerant and excluding species. No differences in Cu accumulation were found between *A. capillaris* and either *Salix* spp. or *P. nigra*, and concentrations never exceeded the toxicity thresholds proposed by Marschner (1995). Shoot Cu concentrations of *A. capillaris* were 2- to 3-fold lower than those determined in grass species (*Festuca* sp. and *Holcus lanatus*) growing sporadically across the mine tailings. The woody tree species used here were found growing on either the Touro site itself, or in a Cu-contaminated wood preservation site (Biogeco experimental station, France). This study therefore confirms that plant colonists such as these in contaminated areas are good candidates for remediation purposes. Previous long-term field experiments showing a successful and sustainable application of SRC within phytomanagement of contaminated sites have mostly targeted the phytoextraction of TE (such as Cd, Zn) over time (French *et al.*, 2006; Hartley *et al.*, 2011; Dimitriou *et al.*, 2012). Here, the two *Salix* spp. and *P. nigra* effectively excluded potentially toxic metals, such as Cu, indicating that phytostabilisation can be successfully combined with excluder-based SRC. Moreover, the biomass produced can be used for bioenergy purposes or feedstock for other applications. For example, Jones *et al.* (2016) showed that biochar produced from the biomass harvested from SRC implemented in phytostabilisation techniques could be successfully used to reduce the mobility and phytotoxicity of Cu in soils from a wood preservation site.

Soil enzymes are highly sensitive to excess concentrations of soluble trace metals and have been recommended as biochemical indicators for assessing evolution in soil quality during the remediation of metal-contaminated soils

(Renella *et al.*, 2006). Hydrolase activities are related to the mineralisation of nutrients such as C (arylesterase, invertase and β-glucosidase), N (urease), P (phosphomononesterase) and S (arylsulphatase) and are considered to be relevant indicators of soil quality (Gil-Sotres *et al.*, 2005). On the other hand, oxidoreductases (catalase and dehydrogenase) are considered indicators of general metabolic microbial activity (Leirós *et al.*, 2000). Microorganisms and soil enzymes are key players in nutrient cycling and soil functionality, but were absent in the highly acidic and Cu-contaminated tailings. The compost addition promoted microbial activity and significantly increased the activities of all assayed enzymes. This stimulation in microbial activity was maintained over the three years of monitoring, suggesting that the effect of compost on metabolic activity is not transitory but likely to last over time. Parallel studies which were carried out at this site using the denaturing gradient gel electrophoresis (DGGE) technique showed that compost application induced important shifts in the bacterial community structure of the mine tailings which became more pronounced with time (unpublished results). The application of organic amendments (such as pig manure, sewage sludge or compost) has previously been shown to increase enzymatic activities in metal-contaminated soils, and enzymatic activity in the amended soils was often correlated with OM content (and nutrients) rather than with metal availability (Alvarenga *et al.*, 2009; Pérez de Mora *et al.*, 2005; Zornoza *et al.*, 2012; Pardo *et al.*, 2014). Here, in the compost-amended soils enzymatic activities were not found to be significantly correlated with $NaNO_3$-extractable metal concentrations, and only invertase activity showed a significant positive correlation with soil C content.

It can be assumed that the increase in microbial activity and in soil enzymatic activity was almost entirely due to an external input, suggesting that the incorporation of compost has introduced not only OM but also active microorganisms and enzymes. Moreover, the enzyme activity was maintained or even increased throughout the whole experiment, suggesting that the compost addition has led to the establishment of stable, active microbial populations, which was accompanied by an increase in the synthesis and liberation of enzymes and probably by the stabilisation of the enzymes themselves through the formation of complexes with organic and/or inorganic soil colloids, which protects the enzymes from degradation by proteases (Burns, 1982). On the other hand, plant growth and activity greatly stimulated the activity of hydrolase enzymes and microbial activity, as revealed by the increased activity of oxidoreductase enzymes, both being

significantly higher in soils under plant covers than in unplanted soils. This may be due to the amelioration of soil physicochemical properties caused by the establishment of such rapidly growing plants which have likely increased the water infiltration, air diffusion and nutrient availability through rhizodeposition. Additional causes could be the release of enzymes by the plant roots, enhanced microbial enzyme synthesis in the rhizosphere (Grierson and Adams, 2000; George *et al.,* 2005), and/or the protective effect on extracellular enzymes by soil OM (Burns, 1982). Pardo *et al.* (2014) found the halophytic shrub *Atriplex halimus* to influence soil enzymatic activity when cultivated in a mine spoil in combination with mature olive mill-waste compost, fresh pig slurry and hydrated lime. Here, the plant-induced stimulation in the activities of all the assayed enzymes was generally higher in soil under *S. viminalis*. Root exudation is a major factor controlling microbial activity and community structure in the rhizosphere (Kozdrój and van Elsas, 2000; Falchini *et al.,* 2003) and the species-specific differences in root exudate composition could explain the differences between the *S. viminalis* and *A. capillaris*. In parallel studies, the DGGE technique showed a clear separation between the bacterial communities associated with unplanted or planted soils. Moreover, bacterial diversity indices based on the DGGE fingerprints also indicated a higher diversity in soils under the *S. viminalis* (unpublished results). Soil bacterial communities are strongly affected by edaphic variables (particularly by pH) but plants are also known to exert a strong influence on their surroundings and shape the bacterial communities in their vicinity (Berg and Smalla, 2009).

Although the beneficial effects of compost addition were observed over the whole duration of the experiment, the mineralisation of OM over time could lead to a progressive loss of these positive effects. For example, compost addition to an abandoned Zn-Pb smelter helped establish a vegetation cover of poplar trees and *A. tenuis*, as well as reducing shoot metal accumulation, but the single application of compost was considered insufficient two years after the experiment begun (Pierzynski *et al.,* 2002). Here, soil nutrients also tended to decrease with time: reductions in exchangeable Ca, K or Mg, total C and N or available P were observed over the three years. However, these reductions were not always significant and did not negatively affect plant growth or nutrition, or microbial activity and soil enzymatic activities. Continuous monitoring will however be necessary if possible needs for re-applications are to be identified. Mench and colleagues have also questioned the sustainability of metal binding by OM and its influence on

plant growth in Cu- and Pb-contaminated soils. These authors demonstrated the need for a second compost dressing at the Biogeco Cu-contaminated site (France) five years after the first application (Mench, unpublished results). Combining organic residues with inorganic waste materials can potentially lead to long-term stabilisation of the soil-plant-microbial system. Alkaline materials can effectively induce metal hydrolysis reactions and/or co-precipitation with carbonates or act as a precipitating agent for metals in the soil solution (Bes and Mench, 2008). Fe-(hydr)oxides or by-products, rich in Fe-oxides, usually induce a reduction in the most labile TE fractions (i.e. soluble and exchangeable) and increase in the reducible fraction (i.e. oxide-bound) (Komárek *et al.*, 2013), and when combined with organic amendments they contribute towards the formation of insoluble metal complexes and stabilisation of organic matter. Ongoing studies at the Touro field site are currently comparing the effects of the mature compost amendment used in this study with technosol mixtures combining organic wastes (anaerobic and aerobic sewage sludge) and inorganic industrial wastes (fly ash from wood bark combustion, foundry sand, Fe (oxihydr)oxides (ferrihydrite-type) and calcium aluminate slags). The effects on plant growth and productivity, and soil quality and functionality, will be monitored over time.

8.5. Conclusion

Overall, this field study demonstrates the potential for low grade composts (based on agricultural and industrial wastes) in combination with Cu-excluding plant species for the phytostabilisation of an acidic heavily Cu-contaminated tailings soil. Strong reductions in labile Cu, alongside improvements in physicochemical properties, fertility and biological activity, were maintained over the three year monitoring period. The use of waste-derived amendments in this type of site, such as the compost used here, represent a practical and environmentally sustainable solution for the valorisation of wastes products and is in accordance with sustainable management practices within current European policies (EU's Zero Waste Policy, 2013). In summary, compost incorporation and, especially the presence of a plant cover, initiated the establishment of biogeochemical cycles in the mine tailings which were previously almost inert.

8.6. References

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Chapter 9

Implementation of (aided) phytoextraction in Pb/Zn mine tailings using hyperaccumulating and high biomass-producing plant species.

ABSTRACT

Phytoextraction cultivates plants which are able to absorb high amounts of trace metals from soils. The process can be aided by the use of organic amendments or the intercropping of plants. A field trial of (aided) phytoextraction was implemented in an abandoned Pb/Zn-mining area in the NW of Spain. The mine tailings were not amended, fertilised with NPK, or amended with composted municipal solid wastes. Replicate sub-plots were planted with two *Salix* spp. (*Salix atrocinerea* and *S. smithiana*) or the Cd/Zn-hyperaccumulator *Noccaea caerulescens. Salix* sp. were planted in monoculture or co-cropped with the N_2-fixing *Alnus glutinosa*, and *N. caerulescens* was planted in monoculture or co-cropped with the leguminous *Lotus corniculatus.*

Compost amendment improved soil properties such as C/N content and fertility, but decreased soil metal availability. Both plant cover and compost addition increased soil enzymatic activities. Compost addition improved plant nutrition and biomass production of the hyperaccumulator. The best-performing *Salix* sp. was *S. smithiana*, which showed the best results in terms of plant survival and growth, and metal accumulation. After 3 years of plant growth, no significant changes in soil total metal concentrations were observed, however, significant reductions in NH_4Cl-extractable Zn concentrations were observed after cultivating *N. caerulescens*. Intercropping the hyperaccumulator with the legume was efficient in increasing shoot metal concentrations and bioconcentration factors.

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9.1 Introduction

The implementation of Gentle Remediation Options (GRO) for soil clean-up is an alternative option to conventional civil engineering-based soil reclamation techniques. Phytoextraction is based on the cultivation of plants which are able to absorb high amounts of trace metals from soils and accumulate them in their harvestable tissues (Vangronsveld *et al.*, 2009; Mench *et al.*, 2010; Kidd *et al.*, 2015; Robinson *et al.*, 2015); while phytostabilisation technologies aim to establish a vegetation cover and promote the *in situ* inactivation of metals by combining the use of metal-excluding plants and soil amendments (Mench *et al.*, 2000; Vangronsveld *et al.*, 2009). GRO are considered to be inexpensive, non-destructive and able to restore soil fertility and ecosystem functions. In addition, these technologies can provide income for local farming communities (Witters *et al.*, 2009; Aronsson *et al.*, 2010; Mench *et al.*, 2010; Ruttens *et al.*, 2011; Van Slycken *et al.*, 2013b). According to Conesa *et al.* (2012) the commercial success of phytotechnologies depends on the production of valuable biomass on contaminated land (such as timber production, bioenergy crops, feedstock for pyrolysis or biofortified products), rather than its application solely as a remediation technique. The use of contaminated land for the production of economic yields falls within the concept of phytomanagement (Robinson *et al.*, 2009). However, only a limited number of studies evaluating the feasibility of GRO under field conditions can be found, and there is a clear need for long-term field experiments monitoring the efficiency of these processes if they are to reach full-scale deployment (Mench *et al.*, 2009; Vangronsveld *et al.*, 2009; Mench *et al.*, 2010; Kidd *et al.*, 2015).

To be effective phytoextractors plants must be able to accumulate large concentrations of metals in their harvestable tissues and have a reasonable biomass production so that soil clean-up by metal removal can be achieved over a realistic time period. One option is to cultivate hyperaccumulating plant species which can accumulate extreme concentrations of metals in their shoots (up to 1-3% DW metal) (Li *et al.*, 2003; Bani *et al.*, 2015). However, the successful application of hyperaccumulators has some limitations, such as their frequently low biomass production, slow growth or limited root development (Keller and Hammer, 2005; Kidd *et al.*, 2015). Fast-growing woody species were proposed as an alternative to hyperaccumulators (Dickinson, 2000; Pulford and Watson, 2003). Members of the family Salicaceae (especially the genus *Salix*) have been shown to accumulate high amounts of trace metals such as Cd or Zn (Unterbrunner *et al.*, 2007;

Wieshammer *et al.*, 2007). Over the last decade, the use of agro-forestry practices such as short rotation coppice (SRC) with high biomass-producing trees has attracted the attention of land managers and policy makers of trace metal-contaminated soils since it can combine bioenergy production, environmental risk attenuation, and restoration of soil fertility and ecosystem services (Van Slycken et al., 2013a). Fast growing woody species, such as *Eucalyptus* spp., *Populus* spp. and *Salix* spp., are grown worldwide for bioenergy purposes under short rotation coppice (SRC) regimes. It has been shown that even on trace metal-contaminated soils high biomass yields can be obtained, with long-term prospects for a positive economic balance (Aronsson *et al.*, 2010; Ruttens *et al.*, 2011; Witters *et al.*, 2012). Willows or poplars are well suited for phytoremediation due to their deep root systems, high transpiration rate, high metal tolerance, and ability to grow on nutrient-poor soils (Dickinson, 2000; Pulford and Watson, 2003). Some species or clones of willow have high bioconcentration factors (BCFs) for Cd (up to 27) and Zn (up to 3) (Dickinson and Pulford, 2005; Wieshammer *et al.*, 2007).

Phytoextraction efficiency can be improved using appropriate agronomic and crop management practices to optimise plant growth and/or metal uptake and bioaccumulation. Improvements in the phytoextraction capacity of metal (hyper) accumulators have been reported after soil inorganic fertilisation (Robinson *et al.*, 1997; Kidd and Monterroso, 2005; Bani *et al.*, 2015). Inorganic fertilisation can also indirectly affect metal availability, through ion exchange reactions, the formation of soluble metal complexes or by acidification (Kidd *et al.*, (2015) and references therein). Ammonium fertilisers frequently cause higher Cd concentrations in crops than nitrate fertilisers as a result of the pH decrease caused by nitrification or plant uptake of NH_4^+ (Grant *et al*, 1999).

On the other hand, the incorporation of organic amendments into degraded or contaminated soils can improve soil physical properties, water infiltration, water holding capacity and structure. Moreover, they contain micro- and macro-nutrients for plant growth, and can also decrease bulk density (Vangronsveld *et al.*, 2009). Addition of these amendments is a common practice to facilitate re-vegetation of degraded sites (Brown *et al.*, 2007; Bes and Mench, 2008; Lord, 2015) and at the same time provides a viable manner of recycling waste products. In a pot experiment, organic amendments were shown to improve the Ni phytoextraction efficiency of various hyperaccumulators (Chapter 5 of this thesis). Plant cropping patterns can also improve plant growth and performance. For example, intercropping of plants (which aims at interspecific belowground interactions)

may result in improved nutrient availability (i.e. through N_2 fixation, transfer of fixed N or mobilisation of P due to rhizosphere acidification) and increased crop yields. In addition, as a consequence of these root interactions, intercropped species may alter metal bioavailability in their rhizospheres (Tang *et al.*, 2012). Thus, cropping patterns can be designed so as to improve plant biomass and nutrition, and/or enhance or mitigate metal availability, uptake and accumulation. Alternative cropping patterns to monoculture can potentially enhance the phytoextraction process. Moreover, they can be of high importance in terms of plant productivity and nutrition, soil protection and quality, enhancing biodiversity, or even in pest control (Facknath and Lalljee, 2000; Lasat, 2000; Mench *et al.*, 2010). Alder is an important pioneer species and is recognised as a soil improving plant, through its symbiosis with N_2 fixing microorganisms (e.g., *Frankia*), ectomycorrhizae and arbuscular mycorrhizal fungi (Tarrant and Miller, 1963; Taleshi *et al.*, 2009). It can effectively colonise nutrient deficient and TE-contaminated soils and mine spoils characterised by low soil fertility (Kidd *et al.*, 2015). Alders can also improve soil P availability (Roy *et al.*, 2007). Several species of *Lotus* show potential for incorporating into GRO due to their world-wide distribution and high adaptation to a number of abiotic stresses, such as metal contamination (Escaray *et al.*, 2012). Candidates described as having good potential for cultivation in degraded or marginal soils include *Lotus corniculatus*, *L. uliginosus*, *L. tenuis*, and *L.* creticus (Escaray *et al*., 2012; Safronova *et al*., 2012).

A field trial was established in 2011 in an abandoned Pb/Zn mine (NW of Spain) to evaluate the phytoextraction efficiency of the woody trees, *Salix viminalis* L. or *S. atrocinerea* Brot., and the Cd/Zn-hyperaccumulator, *Noccaea caerulescens* F.K. Mey (formerly *Thlaspi caerulescens* J &C Presl.). The effect of the fertilisation regime (untreated, inorganic fertilisation (NPK) or amendment with composted municipal solid waste) and plant cropping pattern (monoculture vs. intercropping with legumes (*Alnus glutinosa* (L.) Gaertn. or *Lotus corniculatus* L.)) on soil physico- and bio-chemical properties, plant growth, nutritive status and metal accumulation were evaluated over two to four years.

9.2 Materials and Methods

Study site

Experimental field plots were established in the abandoned Pb/Zn mine of

Rubiais in the Lugo province of NW Spain (UTM 29T 660781/4726800). The altitude is 1070 m and the climate of the region is Oceanic, with a mean annual precipitation of 2000 mm and annual temperature of 8–9ºC (Carballeira *et al.*, 1983). The geological substrate and characteristics of the tailings were previously described by Arias-Prieto (1991), Becerra-Castro *et al.* (2012) and Monterroso *et al.* (2014). The mine tailings cover an area of around 30 ha. Metal deposits are rich in sulphides, principally in the form of sphalerite (PbS) and galena (ZnS) in a 7 to 1 ratio. The surrounding area is characterised by the presence of sandstone, quartzite and slate, which alternate with calcite and dolomite. Alumi-umbric Leptosols and Regosols are the most frequent natural soils in the area (IUSS Working Group WRB, 2006).

Experimental design and set-up

The field trial was carried out in two adjacent areas of the mine tailings; one area was used for cultivating woody trees and one for the Cd/Zn-hyperaccumulator. For the woody trees, the area was ploughed (the first 30 cm of surface soil) and divided into two blocks, R and RC, with a surface area of approx. 400 m^2 each. Block R was not amended and block RC was amended with mature organic compost in September 2011 at an addition rate of 5% w/w. The compost was elaborated from municipal solid wastes and bark chippings by the local company Tratamientos Ecológicos de Noroeste (TEN s.l.) which is dedicated to the recycling of waste products and manufacturing of organic fertilisers and soil improvers. The physicochemical properties of the compost are given in Table 9.1. The compost was mechanically applied, and the soil was tilled after compost addition to a depth of 0-30 cm. Both blocks (either non-amended (R) or compost-amended (RC)) were divided into sub-plots of 5m x 5m, with 1m spacing between sub-plots. Six-month-old rooted cuttings of *Salix smithiana* BOKU 03 CZ-001 and *S. atrocinerea* were planted in monoculture or in inter-cropping with *Alnus glutinosa* four weeks after compost incorporation. Three random replicate sub-plots were established for each plant species/cropping pattern in each block. Woody species were planted with a 0.80 m spacing to yield a planting density of 10000 cuttings ha^{-1} in monoculture and 7000 cuttings ha^{-1} in intercropping. The cuttings of *S. smithiana* and *S. atrocinerea* were obtained from plants occurring naturally in metal-contaminated sites and both were previously described as good metal accumulators (Dos Santos Utmazian and Wenzel, 2007; Moreno-Jiménez *et al.*, 2009). Plants of *A. glutinosa* were obtained from a local tree nursery.

In April 2012, non-amended subplots planted with *S. smithiana* and *S. atrocinerea* monoculture were subdivided and NPK fertilisation was applied to half of the subplot during the following two years (150 kg P ha^{-1} (as P_2O_5), 200 kg K ha^{-1} (as KCl) and 100 kg N ha^{-1} (as NH_4NO_3)). N was incorporated in a split application of 50 kg ha^{-1} each, with an interval of 1 month between each addition. These plots are referred to as NPK-fertilised non-amended, while non-fertilised plots are referred to as untreated non-amended soil.

A smaller area was used to evaluate the performance of the Cd/Zn-hyperaccumulator. As above, the area was ploughed and divided into two blocks, RP and RPC, with a surface area of approx. 30 m^2 each. Block RP was not amended and block RPC was amended with mature organic compost in April 2012 at an addition rate of 5% w/w. Both blocks were divided into sub-plots of 1m x 1m, with 0.5m spacing between sub-plots. Seed of *Noccaea caerulescens* were obtained from a population growing in an abandoned Pb/Zn in Mežica (Slovenia). The site was previously described by Unterbrunner *et al.* (2007). Seeds were germinated in potting culture in the greenhouse and 3-month-old plantlets were transplanted into the field four weeks after compost incorporation in monoculture or intercropped with the metal-tolerant leguminous *Lotus corniculatus*. *Lotus corniculatus* is found growing naturally at the Piedrafita mine site and plants were propagated in the greenhouse from seed collected at the site. Monoculture plots of *N. caerulescens* were established with a density equivalent to 400000 plants ha^{-1}, while for the intercropping the two species were planted so as to maximise positive belowground root interactions between the hyperaccumulator and the legume (with a final density of *N. caerulescens* equivalent to 250000 plants ha^{-1}).

Non-planted sub-plots were established in both experimental areas to evaluate the effects of plant growth and activity on soil physico- and bio-chemical properties. Weed control was carried out mechanically throughout each growth season. The set-up of the experimental plots, incorporation of the compost amendment and evolution in plant growth can be seen in Plate 9.1 and Plate 9.2.

Soil sampling and analysis

Soil samples (five samples per sub-plot from the upper 20 cm layer) were taken with an unpainted stainless steel spade to determine the baseline trace metal content and general physicochemical properties across both the experimental plots (R/RC and RP/RPC) before amendment addition/planting (T=0) and one month

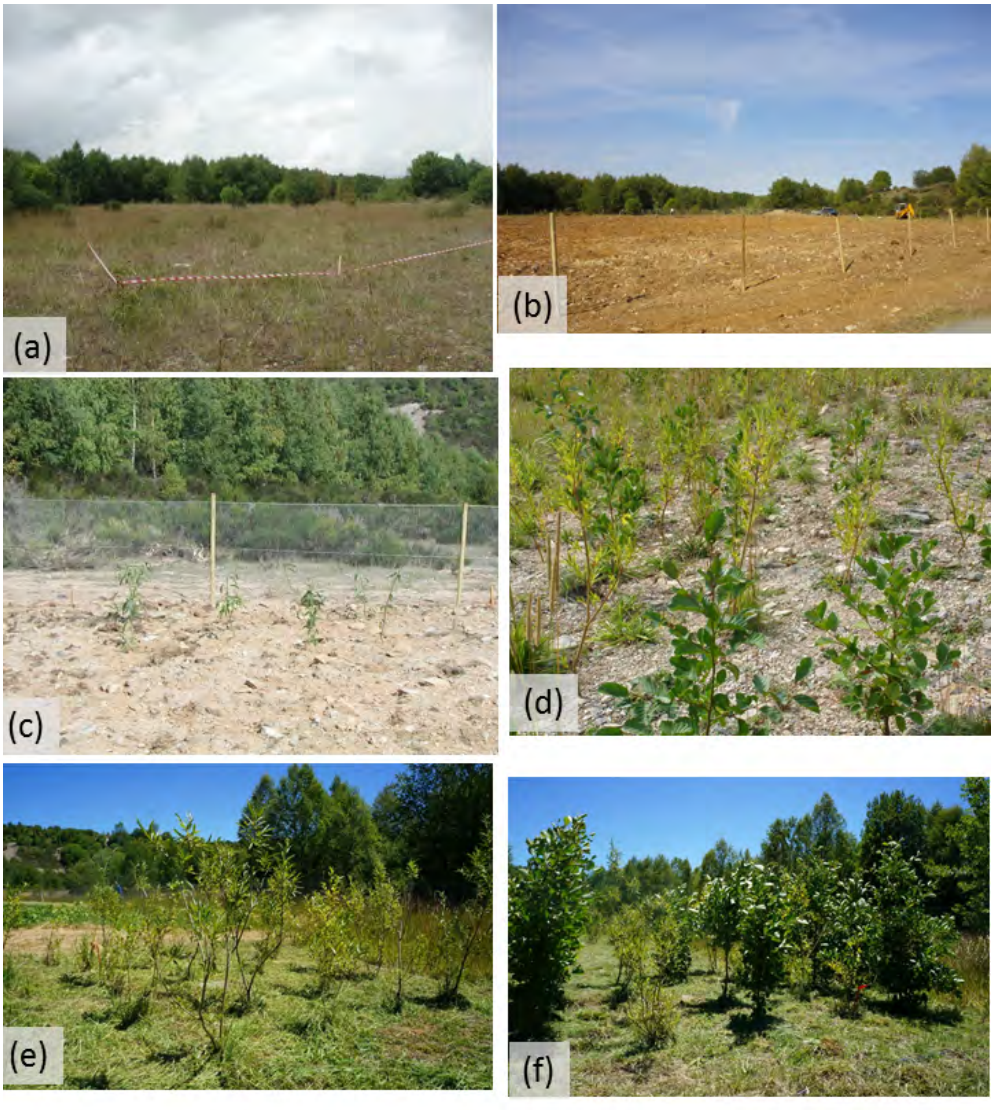


Plate 9.1. Implementation of the (aided) phytoextraction field trial in the mine tailings using high biomass woody species. (a-b) Mine tailings before the experiment was set-up, (c) planting of woody tree species, (d) growth of *S. smithiana* + *A. glutinosa* after 1 year, (e-f) growth of *S. smithiana* in monoculture and intercropped with *A. glutinosa* after 4 years.

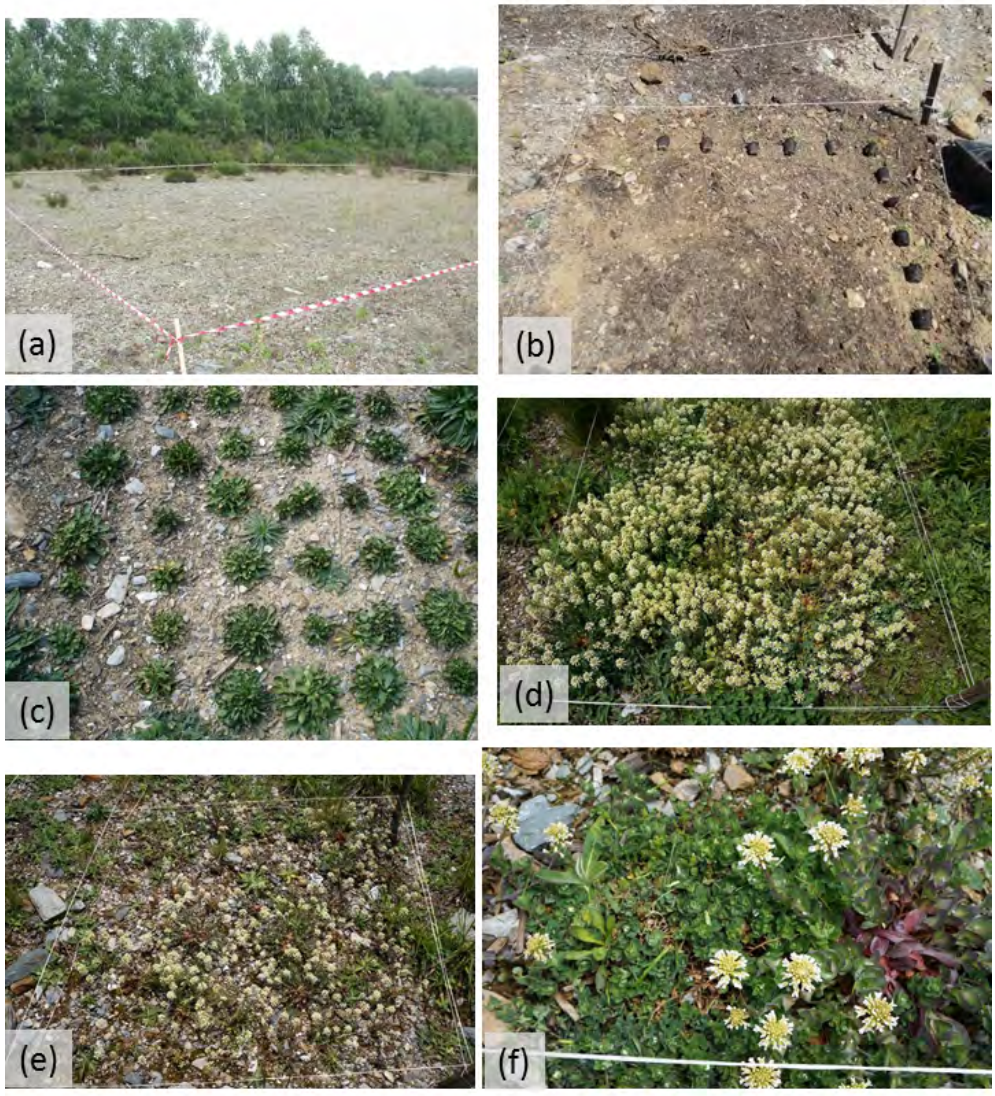


Plate 9.2. Implementation of the (aided) phytoextraction field trial in the mine tailings using hyperaccumulators. (a) Mine tailings before the experiment was set-up, (b) planting of *N. caerulescens* (c) growth of *N. caerulescens* in compost-amended plots 6 months after planting, (d) growth of *N. caerulescens* in compost-amended plots after one year, (e) growth of *N. caerulescens* in non-amended plots after one year, and (f) growth of *N. caerulescens* intercropped with *L. corniculatus* in compost-amended plots after one year.

after amendment incorporation (in RC and RPC). To evaluate plant-induced changes in the physicochemical properties soils were then sampled at time 1.5 and three years after planting (in the case of *N. caerulescens* and *Salix* spp. respectively).

For physicochemical analyses, soil samples were air-dried and sieved to <2 mm. Soil pH was measured in H_2O and 0.1 M KCl using a 1:2.5 (w/v) ratio. Total C and N were analysed by combustion with a CHN analyser (Model CHN-1000, LECO Corp., St Joseph, MI). Available P was extracted with Olsen´s reagent (0.5 M $NaHCO_3$ adjusted to pH 8.2, 1:20 w/v), and determined colorimetrically using the molybdenum blue method (Murphy and Riley, 1962). Soils (0.5 g) were digested in a 3:1 mixture of concentrated HNO_3:HCl after the destruction of organic matter with 3 mL H_2O_2 in Teflon PFA vessels in a microwave accelerated reaction system (MarsXpress; CEM Corp., USA) and the total concentrations of metals were analysed by inductively coupled plasma optical emission spectrometry (ICP-OES, model Vista-PRO, Varian). A certified soil standard (ISE 979 Rendzina Soil) was used to ensure quality of metal quantification. Exchangeable cations (Ca, Mg, Al, Na and K) and metal availability (Cd, Pb and Zn) were estimated through extraction with 1M NH_4Cl (1:20 w/v 16h equilibration) and measured by ICP-OES.

Metal fractionation was assessed following a modified version of the three step BCR sequential extraction procedure (Rauret *et al.*, 2000). The BCR procedure was modified to separate exchangeable metals (extracted with 1 M NH_4Cl, 16 h equilibrium) and carbonate-bound metals (extracted with 0.11 M CH_3COOH, 16 h shaking), due to the importance of this latter phase in this soil. Five soil fractions were obtained: F1 corresponds with the water-soluble and exchangeable metal fraction (exchangeable), F2 with the acid-extractable metal fraction (carbonate-bound), F3 the reducible (iron and manganese oxide bound forms), F4 the oxidisable fraction (organically bound and sulphide metals) and finally, F5 represents the residual fraction (silicate-bound metals).

Soil biochemical properties were monitored in collaboration with the Soil Enzymology Group of the IIAG (CSIC). Fresh soil samples were collected after 1, 2 and 3 years (for *Salix* spp.) and after 0.5 and 1.5 years (for *N. caerulescens*), sieved to <2 mm and stored at 4ºC for biochemical analyses. Enzymatic activities were determined in the non-amended and compost-amended soils of unplanted plots and plots planted with *S. smithiana*, *S. smithiana* + *A. glutinosa*, *S. atrocinerea*, *N. caerulescens*, or *N. caerulescens* + *L. corniculatus*.

Dehydrogenase activity was determined as described by Camiña *et al.* (1998), and the activity was expressed as µmol INTF g^{-1} h^{-1}. Catalase activity was determined according to Trasar-Cepeda *et al.* (1999) and expressed in mmol H_2O_2 consumed g^{-1} h^{-1}. Urease activity was determined as described by Nannipieri *et al.* (1980), except that the released NH_4^+ was measured with an ammonia electrode. The activity was expressed in µmol NH_3 g^{-1} h^{-1}. Invertase activity was determined following the method described by Schinner and von Mersi (1990). Acid and alkaline phosphomonoesterase activity were determined at pH 6.5 and 10.0, respectively, with 16 mM *p*-nitrophenyl phosphate as a substrate following the method of Tabatabai and Bremner (1969), but with some modifications. Modified Universal Buffer was used to maintain the pH of the reaction mixture as described by Trasar-Cepeda *et al.* (1985). After the incubation (30 min) 2 M $CaCl_2$ was added (to prevent dispersion of soil colloids and to avoid the brown coloration caused by organic matter) and the liberated *p*-nitrophenol was extracted with 0.2 M NaOH. The enzymatic activity was quantified by reference to calibration curves corresponding to *p*-nitrophenol standards incubated with each soil under the same conditions as for the samples (Saá, 1995). ß-glucosidase activity was determined as described for acid phosphomonoesterase activity except that the substrate was 25 mM *p*-nitrophenyl-ß-glucopyranoside the pH was maintained at pH 6.0 and the *p*-nitrophenol released was extracted with 0.1 M THAM-NaOH at pH 12 (Eivazi and Tabatabai, 1988). Arylsulphatase activity was determined following the method described by Tabatabai and Bremner (1969), but using 2 M $CaCl_2$ instead of 0.5 M $CaCl_2$ and quantifying the enzymatic activity by reference to calibration curves corresponding to *p*-nitrophenol standards incubated with each soil under the same conditions as for the samples (Saá, 1995). For the latter four enzymes, the enzymatic activity was expressed in µmol *p*-nitrophenol g^{-1} h^{-1}. For each soil sample, all determinations were made in triplicate and the average values were expressed on an oven-dried soil basis.

Monitoring of plant survival, growth and ionome

For woody trees, the maximum shoot height of trees and tree mortality was measured 2 (2013) and 4 years (2015) after planting. Dead trees were replaced during the first six months. To monitor the plant nutritive status and shoot metal accumulation, sub-samples of the plant aerial biomass (stem and leaves) were collected throughout the growth season and in consecutive years. Plant material was carefully washed with pressurised tap water followed by deionised water,

oven-dried at 45 °C and ground. Dried plant material (approximately 0.1 g) were digested in a 2:1 concentrated HNO_3:HCl mixture on a hot plate at 120 °C, and the concentrations of P, K, Ca, Mg, Fe, Cd, Cu, Mn, Ni, Pb and Zn were measured by ICP-OES. The Cd and Zn bioconcentration factor (BCF) was calculated as the concentration of each metal in plant tissues divided by the exchangeable fraction of metal in soils (using the NH_4Cl-extractable metal concentration). Since phyto-extraction aims to reduce the labile fraction of metals in the soil ("bioavailable stripping") we considered the available metal fraction (in this case the NH_4Cl-extractable concentration) to give a better estimation to the plant's ability to concentrate metals in the aerial tissues.

For *N. caerulescens*, the rosette diameter was measured 0.5 (2012) and 1.5 years (2013) after planting. Plant mortality was determined at each measuring period. Sub-samples of the plant aerial biomass (leaves, peduncle and flowers) were collected from each sub-plot throughout the first growth season and at harvest. Plant material was washed, acid digested, and the concentrations of P, K, Ca, Mg, Fe, Cd, Cu, Mn, Ni, Pb and Zn, and Cd/Zn BCF were determined as above. The total shoot biomass produced in each sub-plot was harvested after 1.5 years (October 2013). Plant biomass was expressed as dry biomass (DW) per m^2 and the total Cd/Zn phytoextracted (metal yield) was calculated as the product of the shoot DW and the shoot metal concentration.

Statistical analyses

At each sampling period, the effects of compost amendment and plant cover on soil physico- and bio-chemical properties, and plant growth and ionome, was assessed using t-student tests or a one-way analyses of variance (ANOVA) followed by a multiple comparison of means using the post-hoc Tukey-test for independent means. When necessary, data were transformed to meet the requirements of ANOVA. The statistical analysis was performed using IBM SPSS Statistics v22 for Windows.

9.3 Results

Effect of compost addition on soil physico- and bio-chemical properties

Table 9.1 lists the mean values (and range) for the main soil properties of the non-amended mine tailings of both experimental blocks before implementing the field trial. The tailings soil presents a neutral to alkaline pH (from 7.1 to 8.6)

Table 9.1. Physicochemical properties of the mine tailings and compost amendment.

	R		RP		Compost
	Mean	**Range**	**Mean**	**Range**	**mean ± *SE***
pH_{H2O}	8.3	7.1-8.9	8.1	7.4-8.1	6.2 ± *0.0*
pH_{KCl}	7.8	6.9-8.3	7.7	7.1-8.4	6.2 ± 0.1
%C	1.31	0.78-2.39	1.22	0.95-1.75	15.4 ± 0.4
%N	0.1	0.06-0.20	0.04	0.01-0.08	1.4 ± 0.0
P olsen (mg/kg)	10.86	5.49-17.6	11.8	7.3-19.5	425.3 ± 33.0
CEC ($cmol_c$ kg^{-1})	19.56	6.58-35.02	24.4	21.1-30.1	57.6 ± 4.5
Al^{3+}	0.03	0.005-0.14	0.023	0.019-0.027	< loq
Ca^{2+}	18.58	6.27-33.3	24.5	20.2-29.2	40.4 ± 1.2
K^{+}	0.12	0.03-0.28	0.058	0.7-1.0	4.4 ± 0.1
Mg^{2+}	0.84	0.28-1.96	0.82	0.70 - 1.00	10.3 ± 0.5
Na^{+}	< loq		< loq		2.5 ± 0.1
Pseudo-total concentration					
g kg^{-1}					
Ca	16.3	4.46 - 40.04	20.1	13.5 - 29.5	30.1 ± 0.9
Fe	45.4	19.1 - 57.7	44	36.5 - 56.8	40 ± 7
K	9.76	4.51 - 16.24	9.15	6.74 - 11.5	5.6 ± 0.2
Mg	4.48	2.77 - 8.51	5.38	4.62 - 6.49	6.8 ± 0.2
P	0.79	0.42 - 1.19	1.18	1.06 - 1.40	11.8 ± 0.3
Al	24.6	10.3 - 37.9	23.8	18.4 - 29.9	31.5 ± 0.4
mg kg^{-1}					
Cd	7.54	4.49 - 29.81	9.99	5.70 - 13.72	1.7 ± 0.2
Co	14.08	7.44 - 21.05	25.1	14.97 - 35.89	12.8 ± 0.5
Cr	18.86	7.19 - 47.19	23.06	14.16 - 59.89	65.0 ± 1.6
Cu	14.08	7.44 - 21.05	41.68	29.54 - 73.78	529 ± 84
Mn	2615	1045 - 3639	2206	1992 - 2514	861 ± 20
Ni	26.6	4.03 - 162.95	12.4	0.20 - 181.5	59.2 ± 8.5
Pb	538	267 - 1307	1053	685 - 1792	85.2 ± 1.4
Zn	3993	2416 - 10175	5246	3159 - 7269	530 ± 17
NH_4Cl-extractable concentration (mg kg^{-1})					
Cd	2.13	0.47 - 5.52	4.13	2.40 - 6.08	<loq
Cu	<loq		<loq		0.66 ± 0.04
Mn	15.17	1.99 - 68.30	15.67	7.13 - 21.23	1.07 ± 0.06
Ni	<loq		0.23	0.16 - 0.35	0.12 ± 0.01
Pb	2.17	< loq - 27.0	4.54	2.31 - 7.26	1.32 ± 0.04
Zn	147	25 - 387	394	179 - 608	1.47 ± 0.17

loq, limit of quantification: 0.04 $cmol_c$ kg^{-1} Na^{+}, 1 mg kg^{-1} Pb, 0.5 mg kg^{-1} Cu, 0.1 mg kg^{-1} Ni and 0.1 mg kg^{-1} Cd.

and low fertility (reflected through the low total C (< 2.4%) and N (< 0.20%) content, and available P (< 20 mg kg^{-1})). The CEC was dominated by Ca, while exchangeable Al, K and Mg were low. Trace element (TE) concentrations were highly variable across the tailings but, in general, elevated concentrations of Cd, Pb and Zn were found (Table 9.1). The mean total concentrations of these TE ranged from 4.49 to 29.81 mg Cd kg^{-1}, from 267 to 1792 mg Pb kg^{-1}, and from 2416 to 10175 mg Zn kg^{-1}. The NH_4Cl-extractable concentration of metals ranged from 0.47 to 6.08 mg Cd kg^{-1}, < 1 to 27.0 mg Pb kg^{-1} and 25 to 608 mg Zn kg^{-1}.

Table 9.2 gives an overview of the changes induced in general soil properties one month after compost amendment. Soil pH decreased significantly in both

Table 9.2. Effect of compost addition (5% w/w) in soil physicochemical properties in the mine tailings soil one month after compost amendment addition (considering the whole experimental plot). Asterisks indicate differences between non-amended and amended soils (p < 0.05).

		R	RC	RP	RPC
		mean ± *SE*		mean ± *SE*	
pH $_{H2O}$		8.24 ± 0.03	7.70 ± 0.04 *	8.09 ± 0.04	6.97 ± 0.15 *
pH $_{KCl}$		7.70 ± 0.02	7.44 ± 0.02 *	7.83 ± 0.07	6.76 ± 0.09 *
%C		1.14 ± 0.06	4.58 ± 0.59 *	1.19 ± 0.05	6.08 ± 0.56 *
%N		0.10 ± 0.01	0.27 ± 0.05 *	0.02 ± 0	0.49 ± 0.06 *
Polsen (mg kg^{-1})		13.1 ± 0.7	87.9 ± 11.8 *	13.5 ± 1.0	271.2 ± 48.8 *
CEC (cmolc kg^{-1})		16.5 ± 0.7	24.1 ± 2.0 *	25.0 ± 0.3	19.7 ± 1.3 *
	Al^{3+}	0.016 ± 0.001	0.024 ± 0.002 *	0.023 ± 0.001	0.016 ± 0.001 *
	Ca^{2+}	15.7 ± 0.7	18.7 ± 1.2 *	24.1 ± 0.3	12.5 ± 0.5 *
	K^{+}	0.12 ± 0.01	1.16 ± 0.23 *	0.04 ± 0	1.32 ± 0.2 *
	Mg^{2+}	0.67 ± 0.02	3.45 ± 0.5 *	0.8 ± 0.01	5.61 ± 0.77 *
	Na^{2+}	< loq	0.71 ± 0.14	< loq	0.38 ± 0.13 *
Pseudo-metal concentration (mg kg^{-1})					
Cd		7.12 ± 0.51	6.02 ± 0.4	12.79 ± 0.27	11.1 ±0.47 *
Pb		438 ± 15	377 ± 25	1245 ± 60	1256 ± 75 *
Zn		3394 ± 127	3098 ± 236	6674 ± 151	6105 ± 267
NH_4Cl-extractable metals (mg kg^{-1})					
Cd		1.2 ± 0.05	0.77 ± 0.08	5.53 ± 0.09	3.57 ± 0.23 *
Pb		< loq	< loq	6.12 ± 0.18	4.00 ± 0.29 *
Zn		77.5 ± 3.8	25.5 ± 2.79	562.5 ± 9	232.2 ± 22.6 *

loq, limit of quantification: 0.04 $cmol_c$ kg^{-1} Na^{+}, 1 mg k^{-1} Pb

experimental blocks: in RC pH_{H2O} decreased from 8.2 to 7.7, and in RPC from 8.1 to 7.0 ($p < 0.05$). An increase in total C and N content was also observed reaching maximal values of 4.58% and 0.27% in RC, and 6.08% and 0.49% in RPC, respectively. Compost addition also induced significant changes in the effective CEC but variable effects were observed in the two blocks; in RC the CEC increased significantly (from 16.5 $cmol_c\ kg^{-1}$ to 24.1 $cmol_c\ kg^{-1}$), while in RPC a decrease was observed (from 25.0 to 19.7 $cmol_c\ kg^{-1}$). In the latter, this decrease was largely caused by a reduction in exchangeable Ca^{2+} (from 24.1 $cmol_c\ kg^{-1}$ to 12.5 $cmol_c\ kg^{-1}$). Available P (P Olsen) also increased significantly after compost addition ($p < 0.05$): $NaHCO_3$-extractable P concentrations were up to 7-fold higher in RC and up to 20-fold higher in RPC compared to the corresponding non-amended soils. The pseudo-total concentration of Zn was not significantly altered after amendment addition, while a slight reduction in the mean total Pb concentration was observed in RC and of Cd in RPC. Compost amendment had an important effect on metal availability and significantly decreased the NH_4Cl-extactable concentrations of Cd, Pb and Zn in both blocks: NH_4Cl-extractable concentrations of Cd and Pb (only in RPC) were reduced by 35-36%, while Zn concentrations were reduced by 67% in RC and 59% in RPC compared to corresponding non-amended plots (Table 9.2).

Figure 9.1 shows the soil metal (Cd, Pb and Zn) fractionation of the non-amended and compost-amended soils before planting. In the non-amended soils, Cd was mainly represented by F1 which corresponds to the water-soluble and exchangeable pool (representing 31% of the total Cd), Zn was mainly associated with carbonates (F2; 39%), and Pb was predominantly in the residual fraction or F5 (37%). The addition of compost had a significant effect on the relative distribution of metals amongst the different fractions. In the compost-amended soil, the F1 fraction of Cd and Zn represented was significantly reduced compared to non-amended soil (2.4- and 5-fold lower, respectively ($p < 0.05$)). A significant reduction in the carbonate-bound fraction of all three metals was observed, and this was especially pronounced in Pb (20-fold lower; $p < 0.05$). In contrast, a significant increase in Cd and Zn associated with Fe and Mn oxides (F3) was found, while for Pb this fraction decreased. A significant increase in the F4 fraction of Pb (OM and sulphates bound) was also observed in the compost-amended soils compared to non-amended soil (1.5-fold higher). Finally, addition of compost to the soils caused an increase in the residual fraction of all three metals, although this was only significant for Cd (2-fold increase, $p < 0.05$).

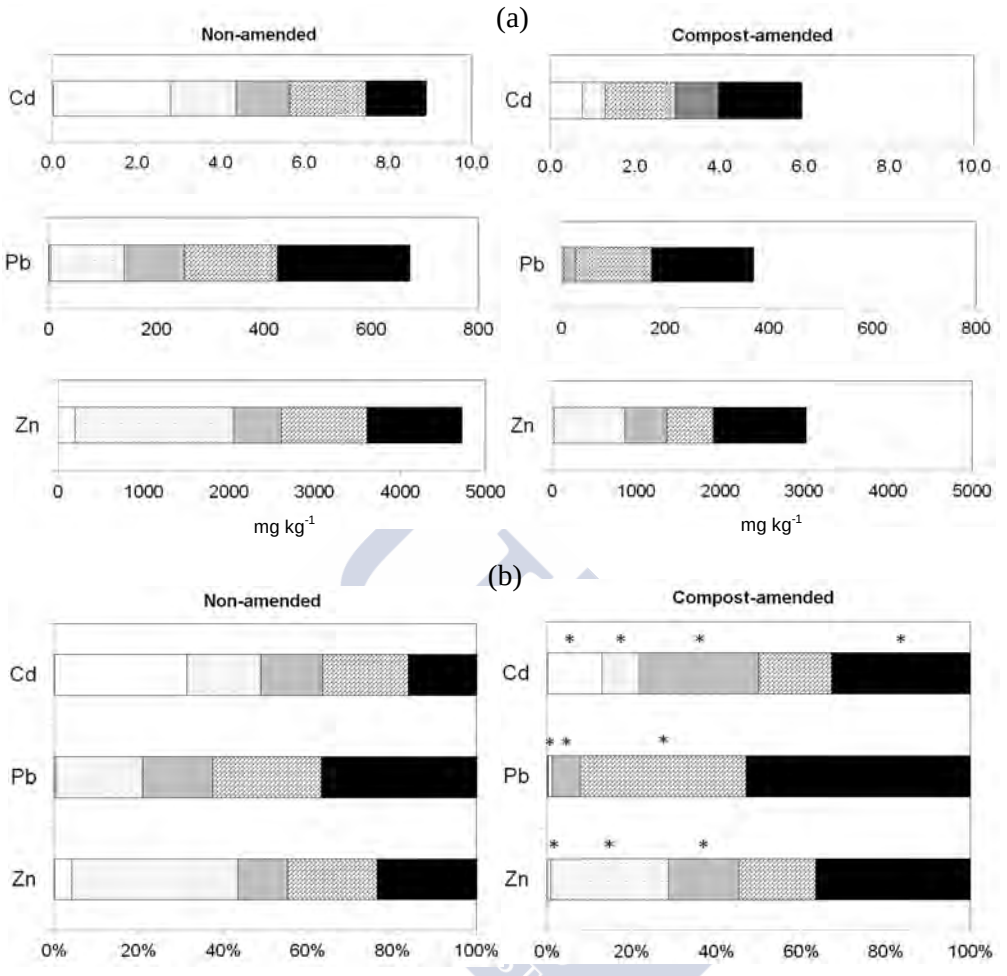


Figure 9.1. Soil metal fractionation in mg kg^{-1} (a) and in percentage (b) in non-amended and compost amended soils before planting. Asterisks indicate significant differences in each fraction between both soils ($p < 0.05$)

The compost addition induced a significant increase in all assayed enzymes, with the exception of catalase (which ranged from 2.78 to 3.01 mmol H_2O_2 consumed g^{-1} h^{-1}). The activity of the oxido-reductase dehydrogenase was 2.1-fold higher in the compost-amended soil than non-amended soil (increasing from 0.12 to 0.25 μmol INTF g^{-1} h^{-1}). For the hydrolase enzymes, activities were increased by 1.5- to 2-fold (depending on the enzyme) after compost addition. Significant increases were observed in invertase (increasing from 3.71 to 5.42 μmol glucose g^{-1} h^{-1}), acid and alkaline phosphomonoesterase (0.28 to 0.52 μmol PNP g^{-1} h^{-1} and 0.47 to 0.81 μmol PNP g^{-1} h^{-1}, respectively), and urease (1.67 to 3.31 μmol NH_3 g^{-1} h^{-1}).

Effects of plant growth on soil physico- and bio-chemical properties

SRC of Salix smithiana and Salix atrocinerea

The effects of plant growth and activity on soil parameters were evaluated after 3 years. Soil C and N content were significantly lower in unplanted soil compared to soil under either *Salix* spp. or cropping pattern. However, this was only the case in the non-amended soil: mean C and N content in unplanted soil was 0.90% and <0.01% and ranged from 1.92-2.19% C and 0.05-0.06% N in planted soils. In the compost-amended soil no significant differences were observed between planted and unplanted soils (C and N content ranged from 4.62 to 5.38% and 0.31 to 0.36%, respectively). Plant growth had no effect on P (P olsen) availability. Pseudo-total concentrations of Cd, Pb and Zn were not significantly modified over the 3 years of plant growth. Figure 9.2 presents the soil pH, CEC, and NH_4Cl-extractable Cd and Zn concentrations in soils under each *Salix* sp. and cropping pattern in either the non-amended or compost-amended plot. Unfortunately, *S. atrocinerea* had a very poor survival rate in some cases mortality was 100%: plants died in intercropped plots in the non-amended soil and in both monoculture and intercropping in the compost-amended soil. Results are therefore not presented for this species in these treatments. In non-amended soil (Block R), the mean soil pH in unplanted plots was 7.9. The presence of *Salix smithiana* (but not *S. atrocinerea*) tended to decrease soil pH (mean pH in untreated plots planted with *S. smithiana* was 7.5) but this decrease was not significant. However, in NPK-fertilised plots soil pH was significantly reduced compared to unplanted plots: the mean soil pH in NPK-fertilised plots planted with *S. smithiana* and *S. atrocinerea* was 7.4 and 7.3, respectively ($p < 0.05$).

On the other hand, plant intercropping did not significantly affect soil pH (mean pH was 7.7 under *S. smithiana* intercropped with *A. glutinosa*) (Figure 9.2). In compost-amended plots no significant difference in soil pH was observed between unplanted plots and soils planted with *S. smithiana* (either in monoculture or intercropped). The woody species did not significantly influence CEC in either soil treatment (non-amended or compost-amended) or cropping pattern. However, plant-induced effects were observed in metal availability, and these were more pronounced in non-amended soil compared to compost-amended soil. In both soil treatments the growth of both *Salix* spp. significantly increased NH_4Cl-extractable Cd and Zn concentrations compared to unplanted soil ($p < 0.05$). In non-amended soil, the highest increases were observed in NPK-fertilised

plots (up to 4.8- and 25-fold higher concentrations of Cd and Zn, respectively). Intercropping of *S. smithiana* had the least effect on metal availability (Cd and Zn concentrations were 2.7- and 8-fold higher in intercropped compared to unplanted soil). In compost-amended soil, a more pronounced effect of tree growth (in this case *S. smithiana*) was observed in intercropped soil (NH_4Cl-extractable concentrations of Cd and Zn were up to 1.8- and 2.6-fold higher in intercropped soil than monoculture).

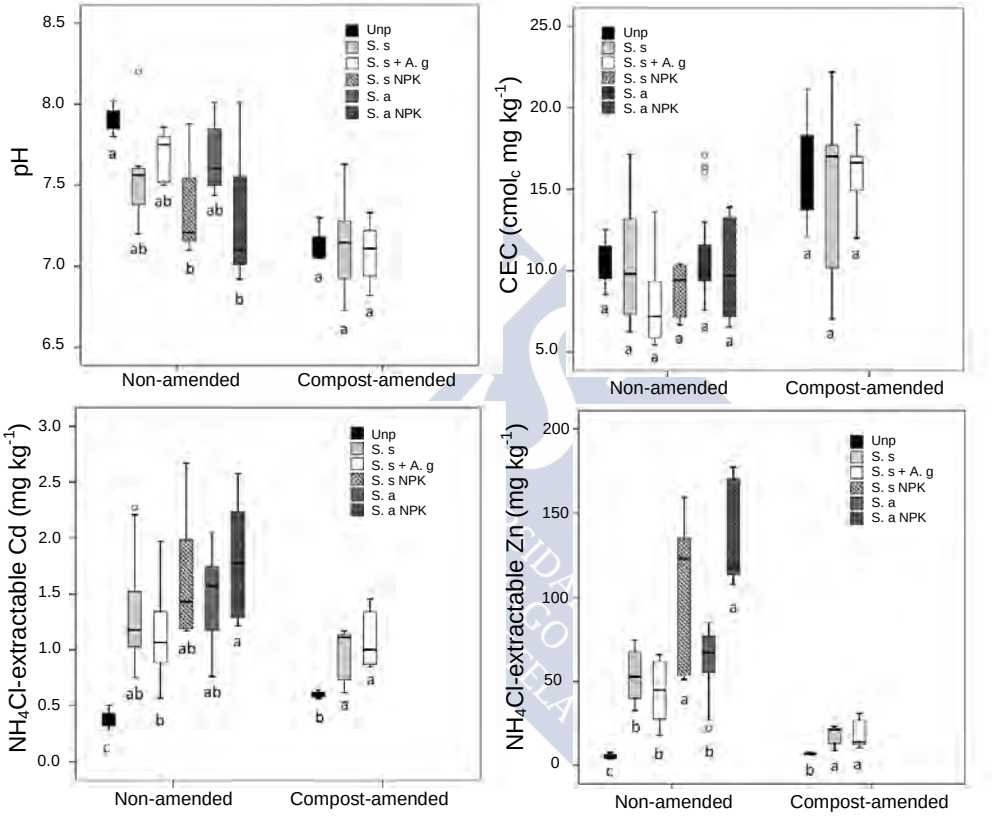


Figure 9.2 Effects of plant species, cropping pattern and fertilisation on soil pH, soil CEC and NH_4Cl-extractable Cd and Zn concentration. Different letters indicate significant differences between plant species and/or treatments in each soil ($p < 0.05$). Boxes represent the median (vertical line) and upper and lower quartiles. Whiskers represent the 90th and 10th percentile. Outliers are represented by unfilled circles. S.s, *Salix smithiana* (untreated); S.s NPK, *S. smithiana* (NPK-fertilised); S.a, *Salix atrocinerea* (untreated); S.a NPK, *S. atrocinerea* (NPK-fertilised); S.s + A.g, *Salix smithiana* + *Alnus glutinosa*.

Soil enzyme activities were monitored over time (sampling events were carried out yearly) in unplanted soils, soils under each *Salix* spp. and cropping pattern in both non-amended and compost-amended soils (Figure 9.3).

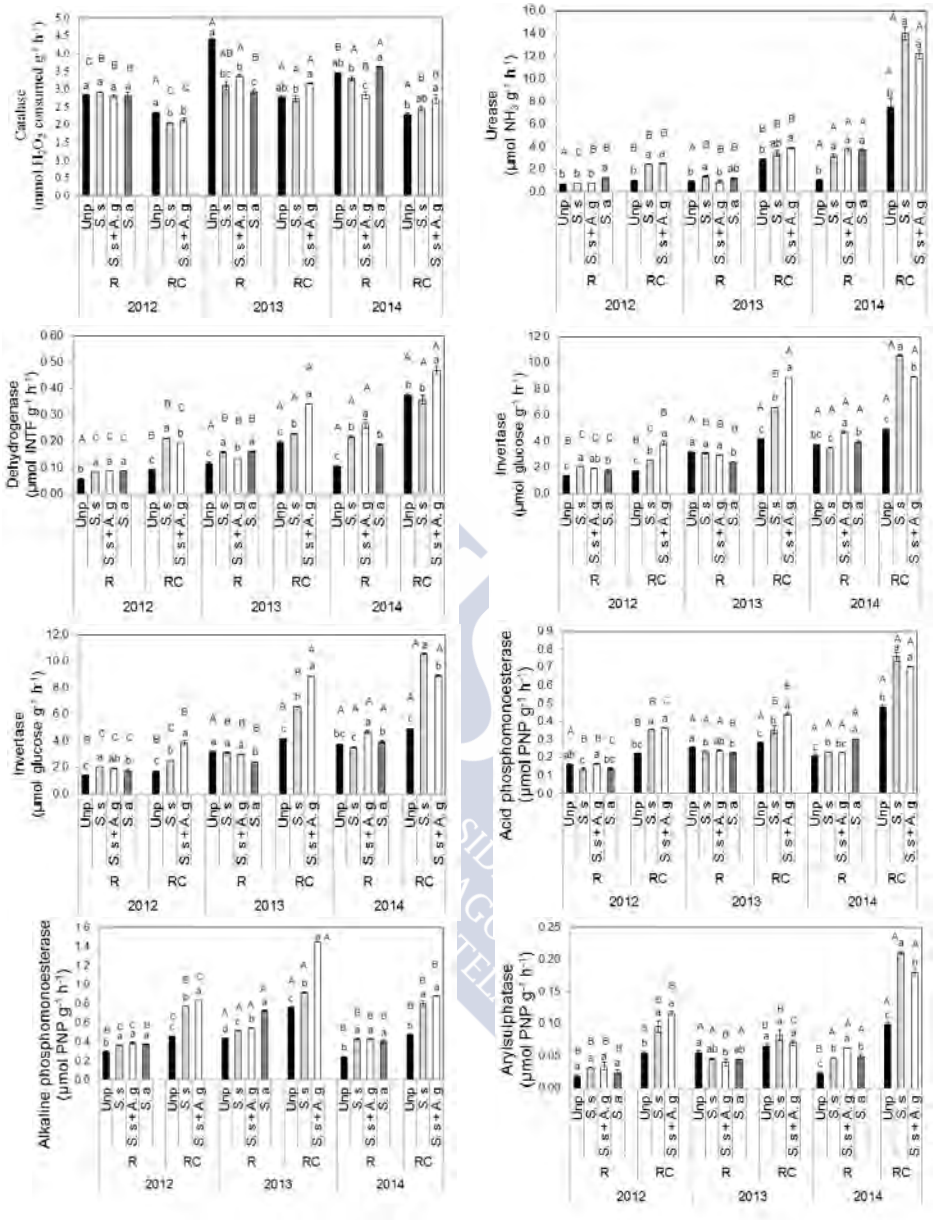


Figure 9.3. Enzyme activities of unplanted soils (Unp) and soils under S.s, *Salix smithiana* (untreated); S.s NPK, *S. smithiana* (NPK-fertilised); S.a, *Salix atrocinerea* (untreated); S.a NPK, *S. atrocinerea* (NPK-fertilised); S.s + A.g, *Salix smithiana* + *Alnus glutinosa* growing in non-amended and compost-amended plots. Lower case letters indicate significant differences between the plant treatments at each sampling time and at the same soil ($p < 0.05$). A capital letter shows a significant difference within the same treatment over time ($p < 0.05$)

Enzyme activities (except for catalase) in compost-amended soil were consistently higher than in non-amended soil at all sampling points. In general, the mean values of enzyme activities were higher in planted soils than in unplanted soils, but this plant effect was highly dependent on the time of sampling. Moreover, enzyme activities in both unplanted and planted soils progressively increased over time, peaking after 2 or 3 years. Activities of catalase, β-glucosidase and alkaline phosphomonoesterase were generally highest after 2 years. On the other hand, dehydrogenase, urease, invertase, acid-phosphomonoesterase and arylsulphatase were highest after 3 years. Pronounced differences between the two *Salix* spp. were not observed: in some cases, and after 2-3 years of growth, enzyme activities were higher in soils under *S. atrocinerea* than *S. smithiana* (e.g. for catalase (2014), invertase (2014), and both acid and alkaline phosphomonoesterase (2014 and 2013, respectively)). Enzyme activities were generally higher in soils under intercropping of *S. smithiana* and *A. glutinosa* than under monoculture of *S. smithiana*. This was observed in dehydrogenase (up to 1.5-fold higher), invertase (1-5-fold higher), β-glucosidase (1.3-fold higher), acid and alkaline phosphomonoesterase (1.3- and 1.6-fold higher, respectively), and arylsulphatase (1.2-fold higher). Moreover, the positive effect of intercropping on enzyme activities was more pronounced in compost-amended soils than in non-amended soils (Figure 9.3).

Cultivation of the hyperaccumulator Noccaea caerulescens

The presence of a plant cover had no significant effect on soil C and N content in the non-amended soil (mean C and N content was 1.29% and 0.02% in unplanted soil, while in planted soils C content ranged from 1.59-1.67% and mean N content was 0.03%). In contrast, plant cover had a strong effect on soil C and N content in compost-amended soil: the mean C and N content in unplanted soil was 1.94% and 0.03%, respectively, and this increased to 7.42-7.80% C and 0.55-0.63% N in planted soils (independent of the cropping pattern). Available P (P olsen) was reduced in non-amended soil in presence of plants ($NaHCO_3$-extractable P concentrations decreased from 14.7 mg kg^{-1} in unplanted soil to 9.7-9.9 mg kg^{-1} in soils cultivated with *N. caerulescens* (again independent of the cropping pattern)). While in compost-amended soil the presence of a plant cover significantly increased available P from 31.4 mg kg^{-1} in unplanted soil to 134.4-154.7 mg kg^{-1} in planted soils. Growth of the hyperaccumulator did not influence soil pH in non-amended soil but significantly reduced soil pH in compost-

amended soil (Figure 9.4). This was the case in both monoculture and intercropping treatments: the mean soil pH was 7.8 in unplanted soil but decreased to 6.8 and 6.7 in soils under monoculture or intercropping, respectively (Figure 9.4). Plant growth induced a reduction in soil CEC in the case of non-amended soils (from 23.0 $cmol_c\ kg^{-1}$ in unplanted plots to 14.2 $cmol_c\ kg^{-1}$ in monoculture of *N. caerulescens* and to 15.7 $cmol_c\ kg^{-1}$ in *N. caerulescens* intercropped with *L. corniculatus*) but did not significantly influence (and even tended to increase) soil CEC in the compost-amended soils (Figure 9.4).

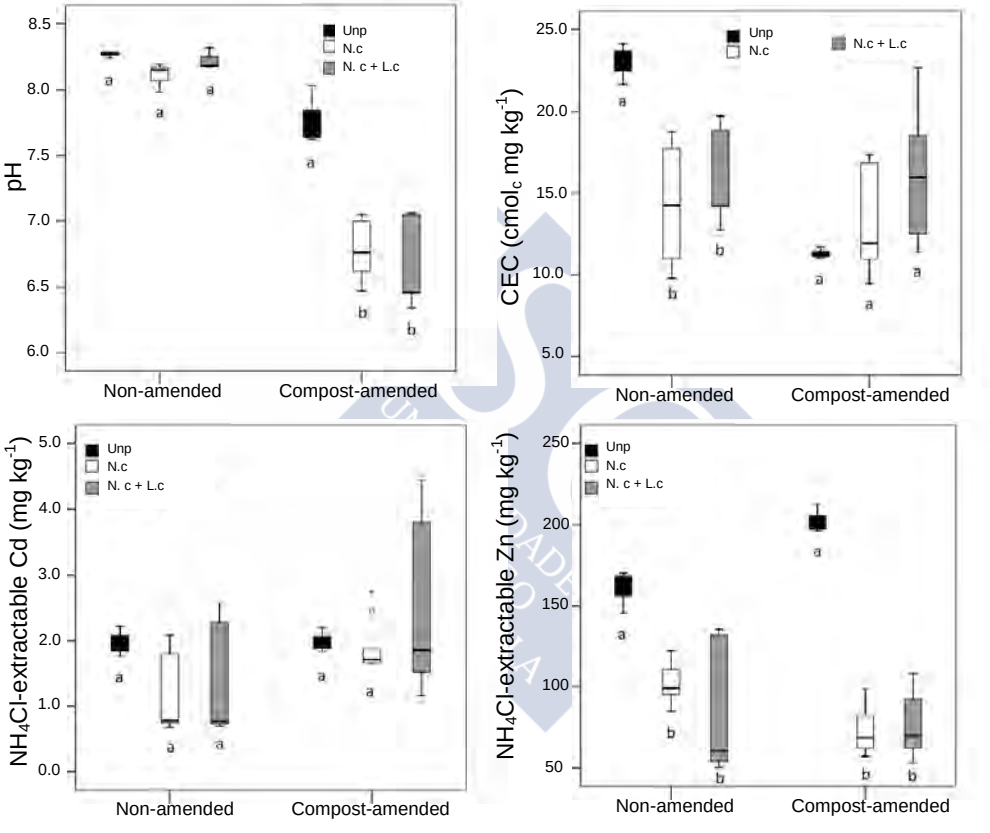


Figure 9.4 Effects of plant species (*Noccaea caerulescens* in monoculture or inter-cropping) on soil pH, soil CEC and NH_4-Cl-extractable Cd and Zn. Different letters indicate significant differences between plant species and/or treatments in each soil ($p < 0.05$). Boxes represent the median (vertical line) and upper and lower quartiles. Whiskers represent the 90th and 10th percentile. Outliers are represented by unfilled circles. N.c, *Noccaea caerulescens*; N.c + L.c, *N. caerulescens* + *L. corniculatus*.

Finally, despite a large variability plant growth did not significantly modify NH_4Cl-extractable Cd concentrations in either non-amended or compost-amended soil (although concentrations tended to be lower in non-amended soil with either

cropping pattern and higher in the intercropping in compost-amended soil). On the other hand, a significant decrease in NH_4Cl-extractable Zn concentrations was observed in both soil types in the presence of a plant cover, and this was more extreme in the compost-amended soil. Mean concentrations of NH_4Cl-extractable Zn decreased from 161 mg kg^{-1} in unplanted soil to 103 mg kg^{-1} in monoculture and 82 mg kg^{-1} in intercropping plots of the non-amended soil, and from 202 mg kg^{-1} in unplanted soil to 74 mg kg^{-1} and 77 mg kg^{-1} in monoculture and intercropping plots of the compost-amended soil ($p < 0.05$). Differences between cropping patterns were not statistically significant (Figure 9.4).

Figure 9.5 presents the soil enzyme activities over time in unplanted and planted soils. In non-amended soil, time did not significantly influence soil enzyme activities, while in compost-amended soil (with the exception of catalase and arylsulphatase) a significant increase was observed with time in activities of both unplanted and planted soils. Except for catalase and arylsulphatase, enzyme activities were generally higher in compost-amended soils, and this was the case at both sampling times (2012 (0.5 years growth) and 2013 (1.5 years growth)). For example, in unplanted soils after 1.5 years the dehydrogenase activity was 3-fold higher in compost-amended than non-amended soil ($p < 0.05$). A similar effect was observed in P cycle enzymes: the activities of β-glucosidase, acid and alkaline phosphomonoesterase were 2.7-, 2.3- and 3.4-fold higher in compost-amended than non-amended soil ($p < 0.05$). Likewise, higher activities of invertase and urease were observed, with differences between these two soil types of up to 3- and 6 -fold. Plant growth also had a significant influence on soil enzyme activities; higher activities were generally observed in planted (independent of cropping pattern) compared to unplanted soils (except for catalase in non-amended soil which was significantly lower in planted soils). Moreover, this plant-induced effect was consistent over time and more pronounced in the compost-amended soil (except for arylsulphatase). No consistent effect of cropping pattern on enzyme activity was found. In compost-amended soil, intercropping had a pronounced effect on urease activity after 0.5 years of growth (increasing from 2.04 μmol NH_3 g^{-1} h^{-1} to 9.09 μmol NH_3 g^{-1} h^{-1}) but this effect was lost after 1.5 years. Similarly, the activities of β-glucosidase, acid and alkaline phosphomonoesterase were increased in soils under *N. caerulescens* when intercropped with *L. corniculatus*, but again this was only observed at the first sampling time. In contrast, dehydrogenase activity was significantly lower in these intercropped soils in both non-amended and compost-amended soil and at both time points. Finally, in non-amended soil similar de-

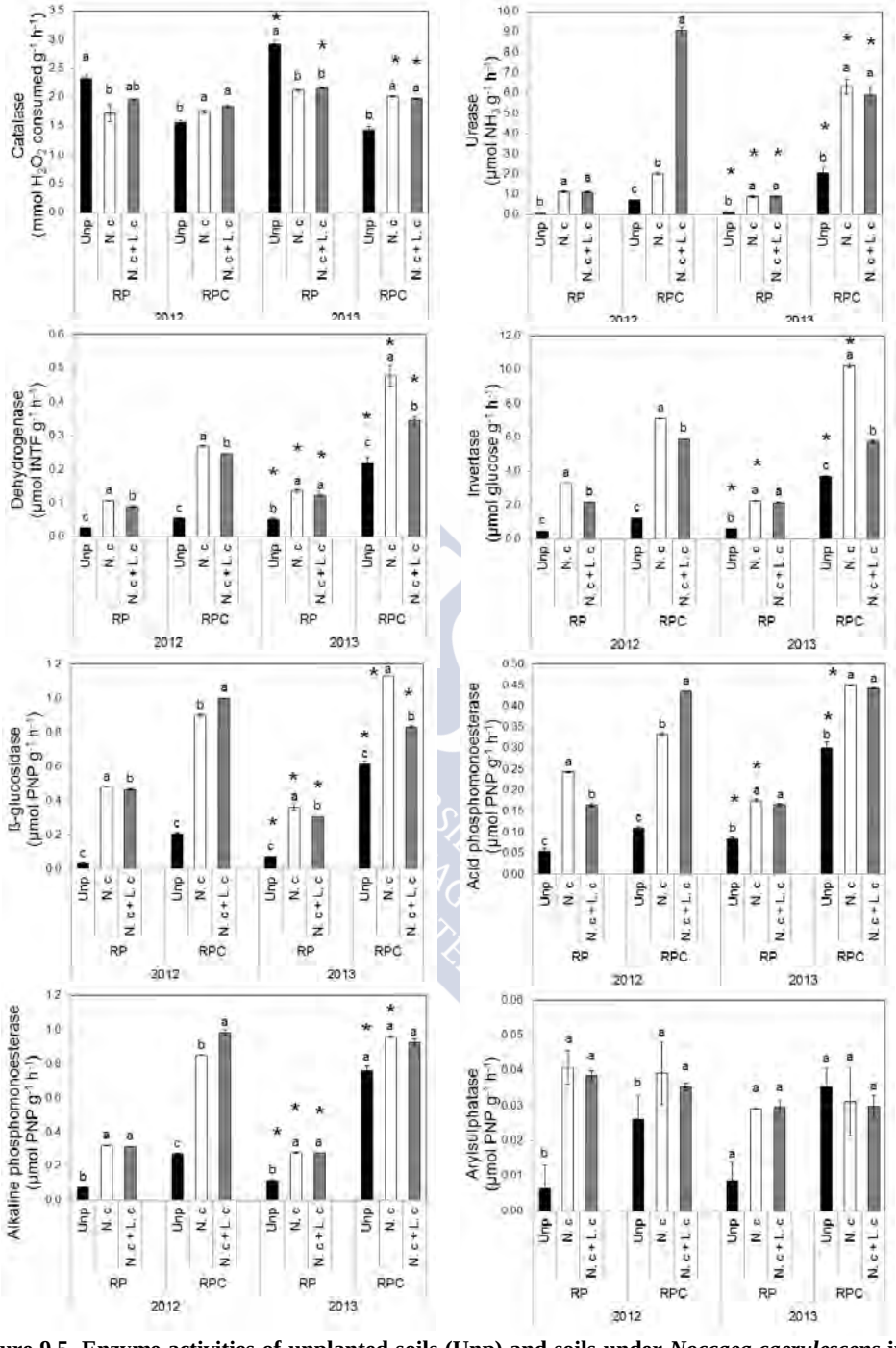


Figure 9.5. Enzyme activities of unplanted soils (Unp) and soils under *Noccaea caerulescens* in monoculture or intercropping and in non-amended (RP) or compost-amended (RPC) plots. Lower case letters indicate significant differences between the plant treatments at each sampling time and each soil ($p < 0.05$). An asterisk indicates a significant difference within the same treatment over time ($p < 0.05$). N.c, *Noccaea caerulescens*; N.c + L.c, *N. caerulescens* + *L. corniculatus*.

creases were observed in β-glucosidase (in 2012 and 2013), acid phosphomonoesterase (in 2012) and invertase (in 2012).

Plant growth, shoot ionome and metal yields

SRC of Salix smithiana and Salix atrocinerea

Plant survival was generally high for *Salix smithiana* over the 4 years in both cropping patterns and soil types: in non-amended soil survival varied from 85 -96%, and in compost-amended soil the survival rate was slightly lower (varying from 72-86%). *S. atrocinerea* only survived in the non-amended soil and in monoculture, where a survival rate of 84% was obtained. The maximum height (per individual) of trees was recorded after 2 (2013) and 4 years of growth (2015) (Figure 9.6). A large variability in plant height was observed between individuals of the same species across the three replicate sub-plots.

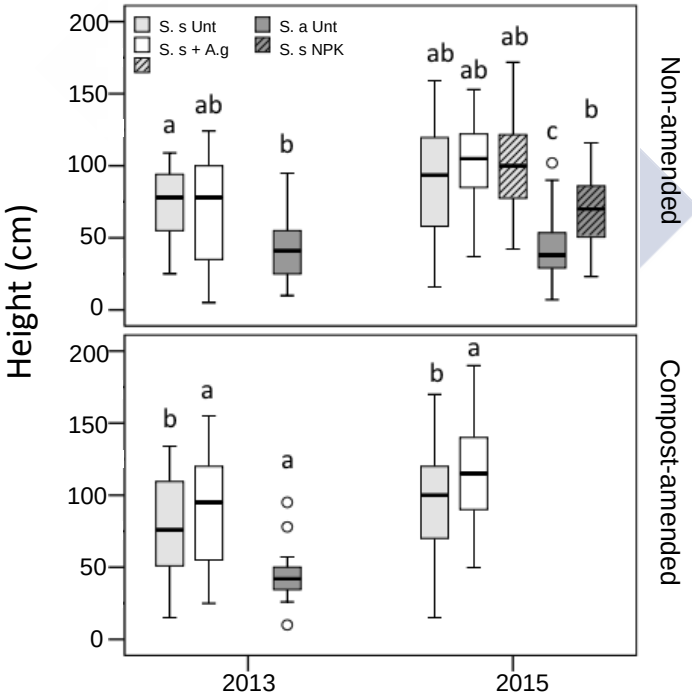


Figure 9.6. Maximum shoot height (cm) of *Salix smithiana* and *Salix atrocinerea* in monoculture and intercropping (only *S. smithiana*) after two and four years growth in non-amended (with and without NPK fertilisation) and compost-amended soils. Different lower case letters indicate significant differences between plant species within the same year ($p < 0.05$).S.s Unt, *Salix smithiana* (untreated); S.s NPK, *S. smithiana* (NPK-fertilised); S.a Unt, *Salix atrocinerea* (untreated); S.a NPK, *S. atrocinerea* (NPK-fertilised); S.s + A.g, *Salix smithiana* + *Alnus glutinosa*.

The maximum height was significantly higher for *S. smithiana* than *S. atrocinerea* at both times: after 4 years of growth the mean height of *S. smithiana* and *S. atrocinerea* was 85.5 cm and 42.5 cm in non-fertilised plots of the non-amended soil (for monoculture; $p< 0.05$). After 2 years plant height was not significantly affected by the NPK fertilisation, but after 4 years a positive effect on plant height was observed (only significant for *S. atrocinerea* ($p < 0.05$); Figure 9.6). NPK fertilisation led to an increase in the mean height of *S. smithiana* from 85.5 cm to 99.4 cm, and of *S. atrocinerea* from 42.5 cm to 69.4 cm. In non-amended soil, *S. smithiana* grown in intercropping with *A. glutinosa* presented a higher mean height than corresponding plants grown in monoculture and without NPK fertilisation (100.7 cm compared to 85.5 cm). However, intercropping did not enhance growth over and above that achieved with NPK fertilisation. In compost-amended soil, tree height was significantly higher in intercropped plots compared to monoculture plots (mean maximum height of 108.3 cm compared to 93.3 cm; $p < 0.05$).

Leaf and stem concentrations of macro- and micro-nutrients in the two *Sali*x species (*S. viminalis* and S. *atrocinerea*) for the different fertilisation treatments (compost-amended, NPK-fertilised, non-amended) and cropping patterns (monoculture and intercropping with *A. glutinosa*) are presented in Tables 9.3a and 9.3b, respectively. Leaf and stem Cd and Zn concentrations are presented in Figure 9.7 and Figure 9.8, respectively.

In general, mean concentrations of Ca, K, Mg, Mn, P and Zn were significantly higher in leaves than in stems ($p < 0.05$), and this was the case for both plant species. In contrast, Cd and Pb concentrations did not differ significantly between leaves and stems ($p > 0.05$). However, the shoot ionome differed between the two *Salix* spp.: *S. smithiana* showed significantly higher leaf Ca, Mg Mn and Zn concentrations, and lower leaf P concentrations, than *S. atrocinerea* when growing in non-amended soils ($p < 0.05$). In compost-amended plots, leaf Ca, Mg and Zn concentrations were again higher in *S. smithiana*, while in this case leaf Mn concentrations were higher in *S. atrocinerea* ($p < 0.05$). *S. atrocinerea* showed higher stem concentrations of P and Cd in non-amended soils and of Ca and P in compost-amended soils. Leaf Ca, Mg, Mn and P concentrations were generally lower in *A. glutinosa* than either *Salix* spp. ($p < 0.05$). Few significant variations in the leaf and stem ionome of each plant species were observed over time.

Table 9.3a. Macro- and micro-nutrient concentrations in leaves of woody tree species (mean concentration ± SE) at the different sampling times. Different lowercase letters indicate differences within the same sampling time and soil treatment (non-amended and compost) ($p < 0.05$). Different capital letters indicate significant differences between different samplings ($p < 0.05$). S.s, *Salix smithiana* (untreated); S.s NPK, *S. smithiana* (NPK-fertilised); S.a, *Salix atrocinerea* (untreated); S.a NPK, *S. atrocinerea* (NPK-fertilised); S.s + A.g, *Salix smithiana* + *Alnus glutinosa*.

			Ca			Fe			K			Mg			Mn			P		
			g kg^{-1}																	
2012	Non-amended	S. s	37.1 ± *2.4*	a	A	0.14 ± *0.01*	a	A	4.16 ± *0.62*	a	C	6.81 ± *0.40*	a	A	0.54 ± *0.05*	a	A	1.11 ± *0.11*	b	C
		S. s + A. g	34.2 ± *1.0*	a	A	0.11 ± *0.02*	a	A	4.17 ± *0.50*	a	D	6.15 ± *0.48*	ab	A	0.47 ± *0.09*	a	A	1.12 ± *0.08*	b	C
		S. a	22.8 ± *1.3*	b	A	0.20 ± *0.07*	a	A	5.90 ± *0.36*	a	B	3.50 ± *0.12*	b	A	0.30 ± *0.02*	a	A	1.82 ± *0.13*	a	B
	Compost-amended	S. s	31.8 ± *1.5*	a	A	0.13 ± *0.01*	a	A	4.27 ± *0.36*	a	C	5.12 ± *0.43*	a	A	0.39 ± *0.03*	a	A	2.75 ± *0.37*	a	B
		S. s + A. g	30.2 ± *1.2*	a	A	0.11 ± *0.01*	a	AB	4.61 ± *0.58*	a	B	4.77 ± *0.20*	ab	A	0.36 ± *0.04*	a	A	2.16 ± *0.25*	a	C
		S. a	25.2 ± *3.4*	a	A	0.16 ± *0.05*	a	A	8.34 ± *0.66*	a	B	3.35 ± *0.40*	b	A	0.47 ± *0.14*	a	A	3.39 ± *0.81*	a	A
2013	Non-amended	S. s	22.5 ± *0.5*	a	B	0.05 ± *0.00*	a	B	5.23 ± *0.19*	a	C	3.98 ± *0.58*	a	B	0.18 ± *0.02*	a	B	1.70 ± *0.08*	a	BC
		S. s + A. g	21.0 ± *2.1*	a	B	0.06 ± *0.01*	a	A	6.12 ± *0.34*	a	CD	4.05 ± *0.37*	a	B	0.17 ± *0.02*	a	AB	1.98 ± *0.15*	a	C
		S. s NPK	18.9 ± *1.6*	a	A	0.04 ± *0.01*	a	B	6.29 ± *1.27*	a	B	3.77 ± *0.54*	a	A	0.16 ± *0.02*	a	AB	1.80 ± *0.20*	a	B
		S. a	10.0 ± *0.1*	b	B	0.09 ± *0.03*	a	A	6.32 ± *0.06*	a	B	2.88 ± *0.20*	a	B	0.11 ± *0.00*	a	B	1.75 ± *0.05*	a	B
		S. a NPK	10.0 ± *0.5*	b	B	0.05 ± *0.00*	a	A	7.86 ± *1.17*	a	B	2.57 ± *0.28*	a	A	0.12 ± *0.01*	a	A	1.87 ± *0.14*	a	B
	Compost-amended	S. s	16.4 ± *0.8*	a	B	0.10 ± *0.02*	a	A	7.61 ± *0.89*	a	BC	3.60 ± *0.18*	a	B	0.18 ± *0.02*	a	AB	3.18 ± *0.10*	a	AB
		S. s + A. g	15.5 ± *0.5*	a	BC	0.11 ± *0.05*	a	AB	6.24 ± *0.19*	a	B	3.32 ± *0.29*	a	B	0.17 ± *0.01*	a	BC	2.65 ± *0.21*	ab	BC
		S. a	13.6 ± *2.1*	a	B	0.09 ± *0.00*	a	A	6.64 ± *0.13*	a	B	2.83 ± *0.43*	a	A	0.26 ± *0.08*	a	A	2.04 ± *0.12*	b	A

			Ca			Fe			K			Mg			Mn			P		
			g kg^{-1}																	
2014	Non-amended	S. s	17.7 ± *0.7*	a	B	0.06 ± *0.00*	a	B	9.41 ± *0.64*	a	B	3.02 ± *0.14*	a	B	0.28 ± *0.08*	a	B	2.38 ± *0.31*	a	AB
		S. s + A. g	16.6 ± *1.1*	ab	BC	0.06 ± *0.01*	a	A	9.46 ± *1.68*	a	B	2.99 ± *0.32*	a	BC	0.29 ± *0.12*	a	AB	2.29 ± *0.32*	a	B
		S. s NPK	15.6 ± *0.6*	ab	AB	0.06 ± *0.01*	a	A	10.66 ± *0.87*	a	A	2.52 ± *0.11*	a	A	0.24 ± *0.05*	a	A	2.50 ± *0.25*	a	AB
		S. a	12.5 ± *1.3*	b	B	0.08 ± *0.02*	a	A	9.42 ± *0.32*	a	A	2.30 ± *0.15*	a	C	0.18 ± *0.07*	a	B	2.52 ± *0.23*	a	A
		S. a NPK	13.6 ± *1.0*	ab	A	0.10 ± *0.01*	a	A	9.85 ± *0.54*	a	AB	2.18 ± *0.23*	a	A	0.20 ± *0.05*	a	A	2.68 ± *0.11*	a	A
	Compost-amended	S. s	18.6 ± *0.9*	a	B	0.08 ± *0.01*	a	A	9.94 ± *0.64*	a	AB	2.94 ± *0.20*	a	BC	0.23 ± *0.03*	a	AB	5.36 ± *0.69*	a	A
		S. s + A. g	20.9 ± *1.8*	a	B	0.14 ± *0.05*	a	A	10.15 ± *1.11*	a	A	3.18 ± *0.25*	a	B	0.24 ± *0.03*	a	AB	4.74 ± *0.84*	a	A
2015	Non-amended	S. s	15.1 ± *0.9*	a	B	0.10 ± *0.05*	a	AB	14.81 ± *3.01*	a	A	2.77 ± *0.22*	a	B	0.08 ± *0.00*	b	B	3.10 ± *0.33*	a	A
		S. s + A. g	14.3 ± *1.0*	a	C	0.07 ± *0.02*	a	A	13.74 ± *0.75*	a	A	2.52 ± *0.10*	a	C	0.07 ± *0.01*	b	B	3.22 ± *0.21*	a	A
		S. s NPK	12.7 ± *0.1*	a	B	0.06 ± *0.00*	a	AB	13.01 ± *0.54*	a	A	2.41 ± *0.22*	a	A	0.09 ± *0.02*	b	B	3.25 ± *0.42*	a	A
		S. a	12.2 ± *1.5*	a	B	0.06 ± *0.01*	a	A	10.87 ± *0.16*	a	A	2.35 ± *0.32*	a	BC	0.08 ± *0.02*	b	B	2.90 ± *0.10*	a	A
		S. a NPK	11.8 ± *0.6*	a	AB	0.08 ± *0.01*	a	A	13.69 ± *0.56*	a	A	1.95 ± *0.04*	a	A	0.12 ± *0.02*	a	A	3.50 ± *0.15*	a	A
	Compost-amended	S. s	10.7 ± *0.2*	a	B	0.10 ± *0.03*	a	A	12.17 ± *0.61*	a	A	1.97 ± *0.07*	a	C	0.05 ± *0.01*	a	B	3.81 ± *0.07*	a	AB
		S. s + A. g	12.3 ± *0.3*	a	C	0.06 ± *0.01*	a	B	12.16 ± *1.64*	a	A	1.81 ± *0.05*	a	C	0.05 ± *0.01*	a	C	3.58 ± *0.33*	a	AB

Table 9.3b. Macro- and micro-nutrient concentrations in stems of woody tree species (mean concentration ± SE) at the different sampling times. Different lowercase letters indicate differences within the same sampling time and soil treatment (non-amended or compost) ($p < 0.05$). Different capital letters indicate significant differences between different samplings ($p < 0.05$).

			Ca			Fe			K			Mg			Mn			P		
			g kg^{-1}																	
2012	Non-amended	S. s	20.0 ± *1.7*	a	A	0.10 ± *0.01*	a	A	4.33 ± *0.28*	a	B	1.55 ± *0.10*	a	A	0.07 ± *0.01*	a	A	1.13 ± *0.05*	b	A
		S. s + A. g	19.9 ± *0.7*	a	A	0.08 ± *0.01*	a	A	4.74 ± *0.25*	a	B	1.49 ± *0.09*	a	A	0.06 ± *0.01*	a	A	1.23 ± *0.06*	ab	AB
		S. a	19.5 ± *1.1*	a	A	0.16 ± *0.07*	a	A	4.70 ± *0.29*	a	B	1.47 ± *0.09*	a	A	0.08 ± *0.01*	a	A	1.40 ± *0.07*	a	B
	Compost-amended	S. s	17.9 ± *1.2*	b	A	0.09 ± *0.01*	b	A	5.53 ± *0.37*	a	A	1.35 ± *0.06*	a	A	0.08 ± *0.01*	a	A	1.30 ± *0.08*	b	B
		S. s + A. g	15.6 ± *1.4*	b	A	0.15 ± *0.02*	b	A	5.76 ± *0.29*	a	A	1.23 ± *0.06*	a	A	0.08 ± *0.01*	a	A	1.28 ± *0.08*	b	AB
		S. a	26.9 ± *1.3*	a		0.29 ± *0.11*	a		7.09 ± *1.02*	a		1.13 ± *0.05*	a		0.06 ± *0.01*	a		2.47 ± *0.13*	a	
2014	Non-amended	S. s	10.2 ± *1.2*	a	B	0.07 ± *0.01*	ab	A	8.60 ± *1.11*	a	A	1.57 ± *0.15*	a	A	0.05 ± *0.01*	a	A	1.42 ± *0.18*	a	A
		S. s + A. g	9.7 ± *0.9*	a	B	0.07 ± *0.02*	ab	A	8.81 ± *1.22*	a	A	1.41 ± *0.16*	a	A	0.06 ± *0.02*	a	A	1.47 ± *0.18*	a	A
		S. s NPK	10.7 ± *1.3*	a	A	0.04 ± *0.01*	b	A	8.94 ± *0.57*	a	A	1.61 ± *0.17*	a	A	0.09 ± *0.05*	a	A	1.70 ± *0.10*	a	A
		S. a	11.0 ± *1.2*	a	B	0.08 ± *0.02*	ab	A	7.84 ± *0.65*	a	A	1.36 ± *0.01*	a	A	0.04 ± *0.01*	a	B	1.98 ± *0.19*	a	A
		S. a NPK	9.8 ± *0.9*	a	A	0.09 ± *0.01*	a	A	7.87 ± *0.35*	a	A	1.44 ± *0.09*	a	A	0.04 ± *0.01*	a	A	1.84 ± *0.25*	a	A
	Compost-amended	S. s	8.6 ± *0.8*	a	B	0.15 ± *0.05*	a	A	7.58 ± *0.84*	a	A	1.19 ± *0.05*	a	A	0.05 ± *0.00*	a	A	1.80 ± *0.03*	a	A
		S. s + A. g	10.4 ± *0.6*	a	AB	0.15 ± *0.04*	a	A	8.20 ± *0.86*	a	A	1.19 ± *0.11*	a	A	0.05 ± *0.00*	a	AB	1.87 ± *0.16*	a	A
2015	Non-amended	S. s	10.3 ± *0.5*	ab	B	0.11 ± *0.01*	a	A	1.51 ± *0.34*	a	C	0.68 ± *0.07*	a	B	0.04 ± *0.00*	a	A	0.60 ± *0.13*	a	B
		S. s + A. g	12.3 ± *0.8*	a	B	0.08 ± *0.01*	a	A	4.04 ± *0.96*	a	B	1.04 ± *0.13*	a	A	0.03 ± *0.01*	a	A	1.01 ± *0.10*	a	B
		S. s NPK	8.2 ± *1.2*	abc	A	0.07 ± *0.01*	a	A	6.05 ± *2.78*	a	A	0.81 ± *0.25*	a	B	0.03 ± *0.00*	a	A	1.00 ± *0.48*	a	A
		S. a	10.5 ± *1.6*	ab	B	0.11 ± *0.01*	a	A	2.46 ± *0.26*	a	C	1.11 ± *0.12*	a	A	0.03 ± *0.00*	a	B	1.00 ± *0.09*	a	B
		S. a NPK	8.1 ± *1.1*	b	A	0.10 ± *0.02*	a	A	2.95 ± *0.60*	a	B	0.93 ± *0.14*	a	B	0.06 ± *0.02*	a	A	1.16 ± *0.24*	a	A
	Compost-amended	S. s	6.5 ± *0.3*	a	B	0.12 ± *0.04*	a	A	0.99 ± *0.25*	a	B	0.60 ± *0.06*	a	B	0.06 ± *0.02*	a	A	0.31 ± *0.06*	a	C
		S. s + A. g	7.0 ± *0.3*	a	B	0.10 ± *0.02*	a	A	2.07 ± *1.22*	a	B	0.64 ± *0.02*	a	B	0.05 ± *0.01*	a	B	0.78 ± *0.39*	a	B

Leaf Zn concentrations of *S. smithiana* significantly declined over time in non-amended plots but not in compost-amended plots (from 3325-4248 mg kg^{-1} in 2012 to 1327-1559 mg kg^{-1} in 2015) (Figure 9.7a).

The most pronounced effects of the cropping treatments on shoot ionome were observed in plants grown in compost-amended soils, but these were dependent on the plant species and the sampling period. NPK fertilisation had little effect on plant nutrient concentrations and tended to reduce shoot Cd/Zn concentrations but this was not statistically significant. In general, compost addition significantly increased leaf K, Mn and P concentrations, and this was independent of the cropping pattern ($p < 0.05$). *S. smithiana* cultivated in compost-amended plots presented significantly higher leaf P concentrations in both monoculture and intercropping plots (by up to 2.5-fold, Table 9.3a; $p < 0.05$). This effect was observed during the years 2012-2014, but was no longer observed at the last sampling period in 2015. Leaf K concentrations were frequently higher in both *Salix* spp. in compost-amended plots (up to 1.5-fold; $p < 0.05$); however, this increase was not consistently observed in both species and was only found during the first two years of growth.

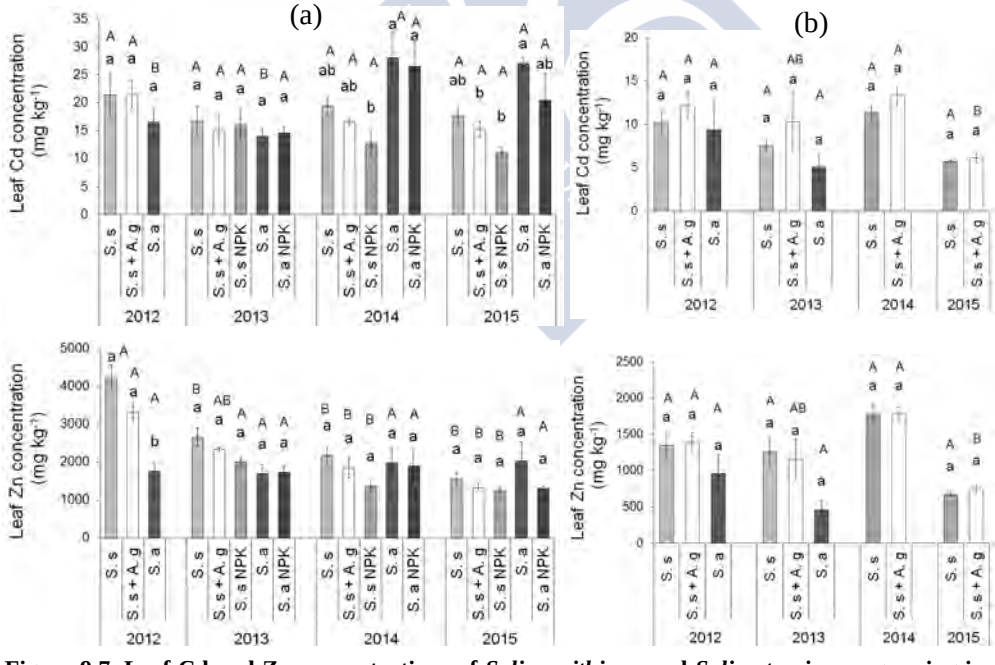


Figure 9.7. Leaf Cd and Zn concentrations of *Salix smithiana* and *Salix atrocinerea* growing in non-amended soils (a) and compost-amended soils (b). Different lowercase letters indicate differences within the same sampling time and soil treatment (non-amended or compost) ($p < 0.05$). Different capital letters indicate significant differences between different samplings ($p < 0.05$). S.s, *Salix smithiana* (untreated); S.s NPK, *S. smithiana* (NPK-fertilised); S.a, *Salix atrocinerea* (untreated); S.a NPK, *S. atrocinerea* (NPK-fertilised); S.s + A.g, *Salix smithiana* + *Alnus glutinosa*.

A significant increase in leaf Mn concentrations was also observed but only for *S. atrocinerea*: leaf Mn concentrations were up to 2.3-fold higher in compost-amended plots compared to non-amended plots in this species ($p < 0.05$). Contrasting effects of compost amendment were observed in stem tissues (Table 9.3b): at some sampling periods K (only in 2012), Fe and P (only in *S. atrocinerea* in 2012) concentrations were significantly increased, while Mg concentrations were generally reduced ($p < 0.05$). After 1 year, stem Ca concentrations of *S. atrocinerea* were also significantly higher when this species was growing in compost-amended plots ($p < 0.05$).

In non-amended soils, the maximum leaf Cd and Zn concentrations were found in *S. smithiana* after 1 year of growth (2012) in both monoculture plots (21.4 mg Cd kg^{-1} and 4248 mg Zn kg^{-1}) and intercroppings (21.5 mg Cd kg^{-1} and 3325 mg Zn kg^{-1}) (Figure 7a). In stem tissues (Figure 9.8a), concentrations peaked for both elements after 3 years in monoculture (16.1 mg Cd kg^{-1} and 1444 mg Zn kg^{-1}) and after 4 years in intercropping (8.5 mg Cd kg^{-1} and 1815 mg Zn kg^{-1}).

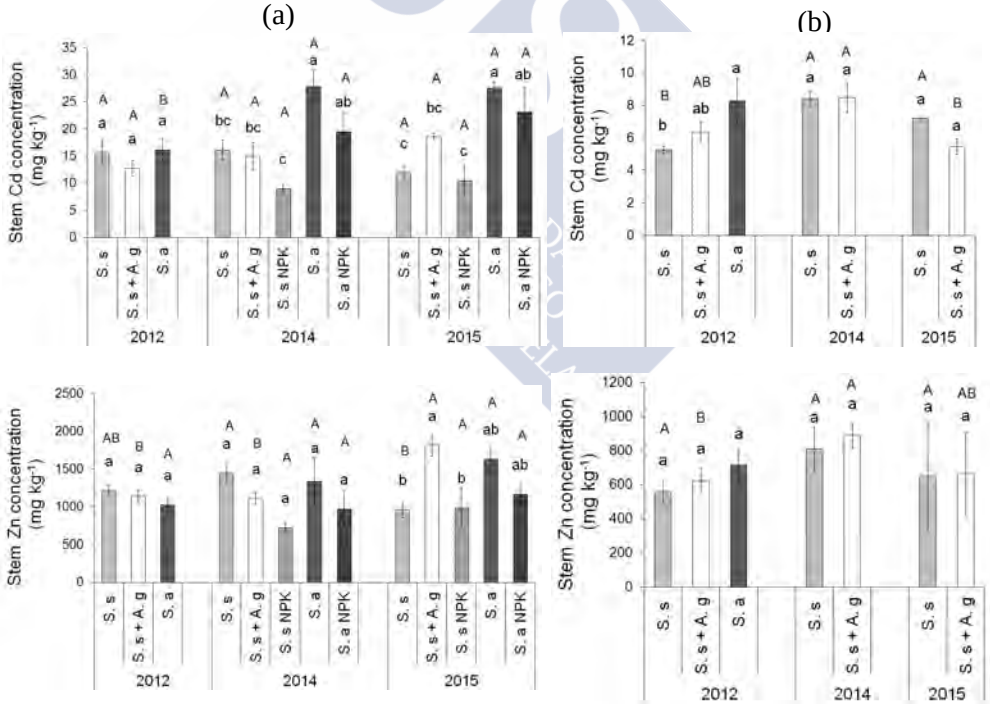


Figure 9.8. Stem Cd and Zn concentrations of *Salix smithiana* and *Salix atrocinerea* growing in non-amended soils (a) and compost-amended soils (b). Different lowercase letters indicate differences within the same sampling time and soil treatment (non-amended or compost) ($p < 0.05$). different capital letters indicate significant differences between different samplings ($p < 0.05$). S.s, *Salix smithiana* (untreated); S.s NPK, *S. smithiana* (NPK-fertilised); S.a, *Salix atrocinerea* (untreated); S.a NPK, *S. atrocinerea* (NPK-fertilised); S.s + A.g, *Salix smithiana* + *Alnus glutinosa*.

Shoot (both leaf and stem) Pb concentrations were consistently below 3 mg kg^{-1}. Compost addition led to a consistent and significant decrease in the concentrations of both Cd and Zn in the leaf and stem tissues of both plant species ($p < 0.05$) (Figure 9.7b and Figure 9.8b). The maximum leaf Cd and Zn concentrations in *S. smithiana* in compost-amended plots were observed after 3 years of growth in both monoculture plots (11.3 mg Cd kg^{-1} and 1782 mg Zn kg^{-1}) and intercroppings (13.3 mg Cd kg^{-1} and 1779 mg Zn kg^{-1}). Similarly, maximal stem concentrations in this species were obtained after 3 years growth (8.4 mg Cd kg^{-1} and 809 mg Zn kg^{-1} in monoculture and 8.5 mg Cd kg^{-1} and 889 mg Zn kg^{-1} in intercropping). Cd and Zn concentrations were 1.2- to 6.9-fold lower (Cd) and 1.1 - to 4.1-fold lower (Zn) in leaves, and 1.7- to 3.4-fold lower (Cd) and 1.3- to 2.7-fold lower (Zn) in stems, in compost-amended plots than non-amended plots. This was observed at all sampling periods (in the case of *S. smithiana*). For example, the mean leaf and stem Cd concentration in monoculture plots of *S. smithiana* at the final sampling period (2015) were 17.5 and 12.0 mg kg^{-1} in non-amended plots and 5.7 and 7.2 mg kg^{-1} in compost-amended plots, respectively. Likewise, the maximum mean leaf and stem Zn concentrations in this species were 4248 mg kg^{-1} and 1444 mg kg^{-1} in non-amended plots and 1782 mg kg^{-1} and 809 mg kg^{-1} in compost-amended plots, respectively.

Cropping pattern had no effect on plant nutritive status, and rarely affected shoot Cd/Zn concentrations. The only significant effect was observed in the stem Cd and Zn concentrations of *S. smithiana* growing in non-amended soils. However, this effect was only observed in the last sampling period (2015): at this time stem concentrations of Cd and Zn were up to 1.6- and 1.9-fold higher when this species was intercropped with *A. glutinosa* (albeit only significant for Zn; Figure 9.8a). Leaf and stem Cd concentrations in *A. glutinosa* were significantly lower than those observed in either *Salix* spp., and were consistently lower than 0.5 mg kg^{-1} in both tissues. Similarly, leaf and stem Zn concentrations were lower than 350 mg kg^{-1} and 500 mg kg^{-1}, respectively.

The Zn BCF for both *Salix* spp. was significantly higher than corresponding Cd BCF values ($p < 0.05$; Figure 9.9). No significant differences were observed between the two *Salix* spp. in the bioconcentration of either metal. In non-amended soils, the Zn BCF was approximately 2-fold higher than corresponding Cd BCF (with the exception of *S. atrocinerea* in NPK-fertilised plots). In compost-amended soils, this difference was even more pronounced, where Zn BCF values were 6.6- to 8.7-fold higher than those of Cd. In non-

amended soils, Cd and Zn BCF were lower in both *Salix* spp. (independent of cropping pattern) when these were fertilised with NPK (albeit not always significantly; Figure 9.9). On the other hand, the mean Zn BCF was higher in *S. smithiana* growing in compost-amended soils than in non-amended soil (mean BCF of 41 compared to either 25 (in non-fertilised plots) and 14 (in NPK-fertilised plots) (based on values for monoculture)). In contrast, Cd BCF values were significantly lower in compost-amended plots compared to non-amended plots. Intercropping of *S. smithiana* and *A. glutinosa* led to an increase in both Cd (only in non-amended plots) and Zn BCF ($p < 0.05$).

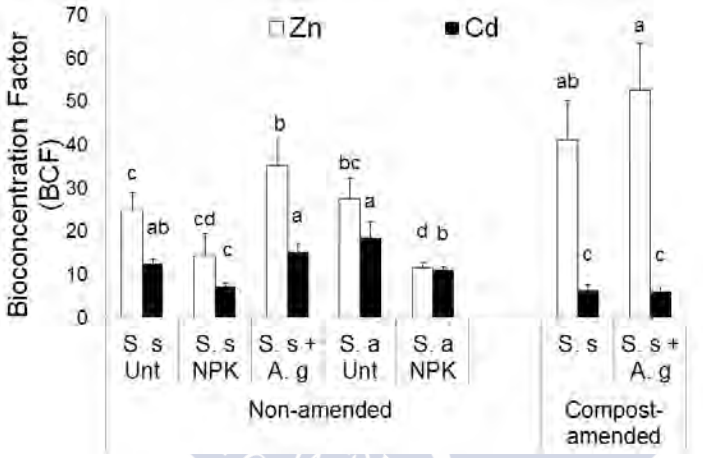


Figure 9.9. Cd and Zn bioconcentration factor (BCF) of *Salix smithiana* and *Salix atrocinerea* growing in non-amended soils and compost-amended soils. Different lowercase letters indicate differences within each metal ($p < 0.05$). S.s Unt, *Salix smithiana* (untreated); S.s NPK, *S. smithiana* (NPK-fertilised); S.a Unt, *Salix atrocinerea* (untreated); S.a NPK, *S. atrocinerea* (NPK-fertilised); S.s + A.g, *Salix smithiana* + *Alnus glutinosa*.

Cultivation of the hyperaccumulator Noccaea caerulescens

Plant survival was high in both non-amended and compost-amended soils, survival tended to be higher in non-amended soil but differences were not significant. In addition, plant cropping pattern did not significantly influence plant survival. In non-amended soil, survival of *N. caerulescens* ranged from 96-98% when grown in monoculture and from 88-96% when intercropped with *L. corniculatus*. In compost-amended soil, survival ranged from 76-98% and 79-88% in monoculture and intercropping plantations, respectively. Figure 9.10 presents the mean rosette diameter of *N. caerulescens* after 1(2012) and 1.5 (2013) years of growth. In non-amended plots, plants reached their maximal size after 1 year, after which time no further increase in growth was found (mean rosette diameter varied from 6.1 to 6.8 cm in monoculture and from 6.1 to 8.0 cm in intercropping).

Compost had a significant effect on plant growth ($p < 0.05$) and this effect was more pronounced with time: after 1 year the mean rosette diameter was 9.9 - 10.8 cm (depending on the cropping pattern) and increased to 20.8 - 21.6 cm after 1.5 years. Cropping pattern did not have a significant effect on plant growth. However, in non-amended soil and after 1.5 years of growth, plants tended to present a larger rosette diameter when intercropped with *L. corniculatus*.

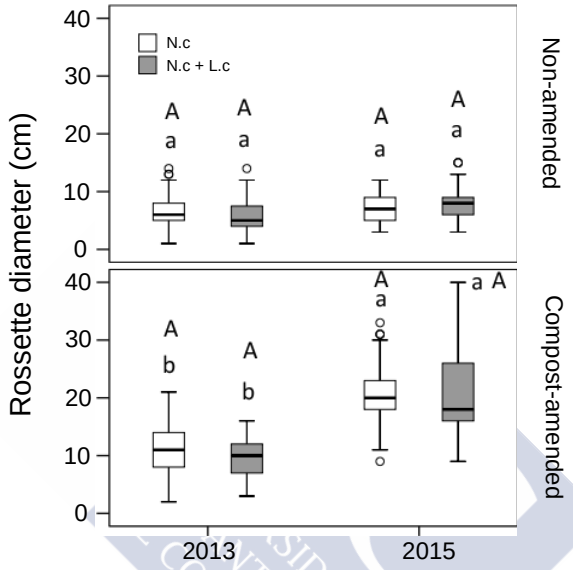


Figure 9.10. Mean rosette diameter (cm) of *Noccaea caerulsescens* grown in monoculture or intercropping after 0.5 and 1.5 years in non-amended and compost-amended soils. Different capital letters denote significant differences between years within each plant species and different lower case letters indicate significant differences between plant species within the same year ($p < 0.05$).

Table 9.4 shows the shoot DW biomass of the hyperaccumulator at harvest when grown in monoculture or intercropped in either the non-amended or compost-amended soil. As for plant growth, shoot DW yields were significantly greater in compost-amended plots than in non-amended plots (2.9- to 4.2-fold higher in monoculture or intercropped plots, respectively). Under monoculture, the mean shoot DW biomass of *N. caerulescens* in compost-amended plots was 210 g m^{-2} (equivalent to 2.1 t ha^{-1}). Shoot DW biomass of *N. caerulescens* obtained from intercropped plots was lower than those of monoculture, but this was only statistically significant in the non-amended soil (1.8-fold lower; $p < 0.05$). *N. caerulescens* presented a mean DW yield in non-amended plots of 1.8 ±0.2 g $plant^{-1}$ when grown in monoculture, and 1.1 ±0.1 g

plant^{-1} when intercropped with *L. corniculatus* ($p < 0.05$). Corresponding yields in compost-amended plots were 5.3 ±0.9 g plant^{-1}(monoculture) and 4.7 ±0.6 g plant^{-1} (intercropping).

Table 9.4. Biomass production (shoot dry weight) and total metal yield of N. caerulescens after two years of growth. Asterisks indicate differences between non-amended and compost-amended soils and ≠ indicate differences between monoculture and intercropping ($p < 0.05$)

		Biomass (g m^{-2})	Zn yield (mg m^{-2})	Cd yield (mg m^{-2})
Non-amended	*N. caerulescens*	72.4 ± 10.0	588.5 ± 86.9	40.6 ± 5.9
	N. caerulescens + *L. corniculatus*	28.2 ± 3.3 ≠	271.4 ± 20.2 ≠	17.9 ± 2.0 ≠
Compost-amended	*N. caerulescens*	210.3 ± 37.7 *	998.4 ± 174.0 *	42.8 ± 6.4
	N. caerulescens + *L. corniculatus*	118.4 ± 14.5 *	751.0 ± 109.0 *	34.0 ± 2.5

Shoot macro- and micro-nutrient concentrations of the aboveground plant tissues (leaves, flowers and peduncle) are shown in Tables 5a-5c. Plant ionome differed significantly between tissues. In non-amended soil, concentrations of Ca, Fe and Mn were higher in leaves (with mean values of 17.7 g kg^{-1}, 1.09 g kg^{-1} and 0.14 g kg^{-1}) than in flowers (9.8 g kg^{-1}, 0.09 g kg^{-1} and 0.07 g kg^{-1}) and/or peduncles (7.7 g kg^{-1}, 0.02 g kg^{-1}, 0.02 g kg^{-1}), while the highest concentrations of P and K were found in flowers (4.4 g kg^{-1}) and the peduncle (24.3 g kg^{-1}), respectively. Similar trends were observed in compost-amended soils: concentrations of Ca, Fe, K, Mg and Mn were highest in leaves (mean values of 15.9 g kg^{-1}, 0.23 g kg^{-1}, 25.6 g kg^{-1}, 3.6 g kg^{-1} and 0.15 g kg^{-1}, respectively), and P concentrations were again highest in flowers (5.3 g kg^{-1}) ($p < 0.05$). The addition of compost had a significant effect on the shoot ionome: an increase in leaf K (1.7-fold), Mg (2.2-fold) and P (2.5-fold) concentrations, flower Mg (1.5-fold), Mn (1.7-fold) and P (1.2-fold) concentrations, and finally, peduncle Mg (1.7-fold), Mn (1.5-fold) and P (1.4-fold) concentrations. On the other hand, Ca concentrations in all three tissues, and leaf Fe concentrations, were significantly lower in plants grown in compost-amended soil compared to non-amended soil ($p < 0.05$). In contrast to what was observed with the woody species, intercropping of *N. caerulescens* with the leguminous *L. corniculatus* had a positive effect on leaf nutrient concentrations. This was observed in all the elements determined and at all sampling times. In general, this effect was more pronounced in the compost-amended soil although a positive effect was also found in the non-amended soil at harvest. Plant

Table 9.5a. Macro- and micro-nutrient concentrations in leaves of *Noccaea caerulescens* (mean concentration ± SE) at the different sampling times. Different capital letters indicate differences between sampling times and asterisks denote significant differences between monoculture and intercropping with *Lotus corniculatus* (p < 0.05). N.c, *Noccaea caerulescens*; N.c + L.c, *N. caerulescens* + *L. corniculatus*.

			Ca		Fe		K		Mg		Mn		P	
			g kg^{-1}											
Before flowering	Non-amended	N. c	16.2 ± *1.33*	AB	0.72 ± *0.06*	B	16.3 ± *0.53*	A	1.79 ±*0.11*	A	0.13 ±*0.00*	B	1.62 ±*0.04*	B
		N. c + L. c	19.8 ± *1.60*	B	0.61 ± *0.04*	B	18.4 ± *1.64*	A	1.84 ±*0.16*	B	0.11 ±*0.01*	BC	1.86 ±*0.21*	A
	Compost-amended	N. c	19.5 ± *1.11*	A	0.22 ± *0.01*	AB	32.3 ± *0.84*	A	5.28 ±*0.25*	A	0.16 ±*0.01*	A	5.58 ±*0.33*	A
		N. c + L. c	20.0 ± *1.34*	A	0.33 ± *0.05*	A	29.4 ± *1.68*	AB	3.98 ±*0.23* *	A	0.13 ±*0.01*	BC	4.54 ±*0.37* *	AB
Early flowering	Non-amended	N. c	18.8 ± *0.82*	A	0.14 ± *0.01*	B	15.7 ± *0.92*	A	1.00 ± *0.03*	BC	0.09 ± *0.01*	BC	1.98 ± *0.05*	A
		N. c + L. c	20.5 ± *0.54*	AB	0.19 ± *0.06*	B	16.2 ± *0.30*	AB	1.41 ± *0.12* *	B	0.12 ± *0.00*	BC	1.90 ± *0.04*	A
	Compost-amended	N. c	12.2 ± *0.71*	BC	0.11 ± *0.01*	AB	14.6 ± *0.36*	C	2.28 ± *0.10*	B	0.10 ± *0.01*	B	3.05 ± *0.11*	BC
		N. c + L. c	16.3 ± *0.58* *	AB	0.25 ± *0.04* *	AB	20.1 ± *0.46* *	C	3.51 ± *0.25*	A	0.22 ± *0.03* *	A	3.62 ± *0.14* *	BC
Mid flowering	Non-amended	N. c	20.2 ± *1.79*	A	0.13 ± *0.03*	B	17.3 ± *1.73*	A	0.63 ± *0.19*	C	0.08 ± *0.00*	BC	0.91 ± *0.01*	D
		N. c + L. c	27.7 ± *4.47*	A	0.21 ± *0.09* *	B	14.7 ± *1.01*	AB	3.09 ± *0.93*	A	0.14 ± *0.04*	B	1.45 ± *0.34*	A
	Compost-amended	N. c	15.1 ± *0.60*	B	0.08 ± *0.00*	AB	21.3 ± *2.28*	B	2.57 ± *0.31*	B	0.12 ± *0.00*	AB	3.31 ± *0.46*	BC
		N. c + L. c	17.8 ± *1.20*	AB	0.21 ± *0.02*	AB	22.7 ± *1.77*	BC	3.54 ± *0.19* *	A	0.20 ± *0.01* *	AB	3.15 ± *0.12*	C
After flowering	Non-amended	N. c	12.7 ± *0.35*	B	0.17 ± *0.02*	B	18.7 ± *1.57*	A	1.07 ± *0.08*	BC	0.05 ± *0.01*	C	1.40 ± *0.03*	BC
		N. c + L. c	14.2 ± *1.33*	B	0.12 ± *0.02*	B	16.8 ± *1.23*	A	1.40 ± *0.15*	B	0.06 ± *0.00*	C	1.16 ± *0.03* *	A
	Compost-amended	N. c	9.8 ± *1.75*	C	0.05 ± *0.01*	B	27.9 ± *2.53*	A	2.36 ± *0.37*	B	0.10 ± *0.01*	B	2.82 ± *0.10*	C
		N. c + L. c	14.2 ± *2.35*	B	0.10 ± *0.02*	B	32.6 ± *4.42*	A	3.77 ± *0.32* *	A	0.13 ± *0.02*	A	3.37 ± *0.29*	BC
Harvest	Non-amended	N. c	13.2 ± *0.17*	B	3.91 ± *0.37*	A	8.6 ± *0.14*	B	1.50 ± *0.09*	AB	0.29 ± *0.02*	A	1.31 ± *0.04*	C
		N. c + L. c	14.9 ± *0.54* *	B	2.61 ± *0.31* *	A	10.4 ± *0.37* *	B	1.85 ± *0.12* *	B	0.21 ± *0.02* *	A	1.53 ± *0.04* *	A
	Compost-amended	N. c	12.0 ± *0.68*	BC	0.29 ± *0.08*	A	27.4 ± *0.46*	A	3.26 ± *0.05*	B	0.12 ± *0.01*	AB	4.34 ± *0.08*	AB
		N. c + L. c	14.3 ± *0.35* *	B	0.36 ± *0.02*	A	29.0 ± *0.70*	AB	3.76 ± *0.10* *	A	0.19 ± *0.01* *	ABC	5.05 ± *0.11* *	A

Table 9.5b. Macro- and micro-nutrient concentrations in flowers of *Noccaea caerulescens* (mean concentration ± SE) at the different sampling times. Different capital letters indicate differences between sampling times and asterisks denote significant differences between monoculture and intercropping with *Lotus corniculatus* (p < 0.05). N.c, *Noccaea caerulescens*; N.c + L.c, *N. caerulescens* + *L. corniculatus*.

			Ca		Fe		K		Mg		Mn		P	
			g kg^{-1}											
Early flowering	Non-amended	N. c	9.0 ± *0.26*	B	0.08 ± *0.00*	A	23.1 ± *0.37*	A	1.33 ± *0.04*	A	0.07 ± *0.00*	A	4.93 ± *0.14*	A
		N. c + L. c	8.4 ± *0.27*	A	0.09 ± *0.01*	A	22.8 ± *0.22*	A	1.18 ± *0.01* *	B	0.07 ± *0.00*	A	4.50 ± *0.14* *	A
	Compost-amended	N. c	7.0 ± *0.46*	B	0.10 ± *0.01*	A	22.8 ± *0.44*	A	1.55 ± *0.06*	B	0.10 ± *0.01*	A	5.77 ± *0.14*	A
		N. c + L. c	5.9 ± *0.48*	B	0.13 ± *0.02*	A	23.5 ± *0.66*	A	1.68 ± *0.08*	B	0.12 ± *0.01*	B	5.95 ± *0.15*	A
Mid flowering	Non-amended	N. c	11.8 ± *0.62*	A	0.08 ± *0.02*	A	16.8 ± *0.73*	B	1.48 ± *0.08*	A	0.08 ± *0.01*	A	3.94 ± *0.07*	B
		N. c + L. c	10.5 ± *0.98*	A	0.10 ± *0.01*	A	16.3 ± *0.57*	B	1.74 ± *0.12*	A	0.07 ± *0.01*	A	3.99 ± *0.17*	B
	Compost-amended	N. c	9.7 ± *0.28*	A	0.05 ± *0.01*	B	13.9 ± *0.49*	B	2.57 ± *0.12*	A	0.09 ± *0.01*	A	4.45 ± *0.33*	B
		N. c + L. c	10.4 ± *0.31*	A	0.08 ± *0.01*	B	14.1 ± *0.32*	B	2.90 ± *0.12*	A	0.17 ± *0.02* *	A	4.86 ± *0.19*	B

Table 9.5b. Macro- and micro-nutrient concentrations in peduncle of *Noccaea caerulescens* (mean concentration ± SE) at the different sampling times. Different capital letters indicate differences between sampling times and asterisks denote significant differences between monoculture and intercropping with *Lotus corniculatus* (p < 0.05). N.c, *Noccaea caerulescens*; N.c + L.c, *N. caerulescens* + *L. corniculatus*.

			Ca		Fe		K		Mg		Mn		P	
			g kg^{-1}											
Early flowering	Non-amended	N. c	7.1 ± *0.48*	A	0.02 ± *0.00*		29.8 ± *0.94*	A	1.65 ± *0.13*	A	0.02 ± *0.00*	A	2.30 ± *0.07*	A
		N. c + L. c	9.6 ± *0.28* *	A	0.02 ± *0.01*	A	25.6 ± *2.56*	A	2.22 ± *0.15* *	A	0.03 ± *0.00* *	A	2.27 ± *0.08*	A
	Compost-amended	N. c	6.3 ± *0.25*	A	0.03 ± *0.01*	A	22.2 ± *1.34*	A	3.80 ± *0.30*	A	0.03 ± *0.00*	A	3.35 ± *0.19*	A
		N. c + L. c	7.3 ± *0.59*	A	0.03 ± *0.00*	A	18.6 ± *1.36*	A	3.20 ± *0.11*	A	0.02 ± *0.00*	A	3.10 ± *0.14*	A
Mid flowering	Non-amended	N. c	6.6 ± *1.01*	A	< loq		19.8 ± *0.29*	B	0.84 ± *0.08*	B	0.01 ± *0.00*	A	1.51 ± *0.24*	B
		N. c + L. c	7.2 ± *0.46*	B	0.03 ± *0.01*	A	19.2 ± *1.52*	A	1.79 ± *0.34* *	A	0.02 ± *0.00*	B	1.67 ± *0.14*	B
	Compost-amended	N. c	5.5 ± *0.16*	B	0.01 ± *0.00*	B	21.4 ± *0.52*	A	2.21 ± *0.17*	B	0.02 ± *0.00*	A	2.68 ± *0.19*	B
		N. c + L. c	4.3 ± *0.32* *	B	0.01 ± *0.00* *	B	20.0 ± *0.61*	A	2.19 ± *0.21*	B	0.03 ± *0.00* *	A	2.31 ± *0.14*	B

loq: 5 mg kg^{-1} Fe

intercropping also showed a beneficial effect on flower and peduncle nutrient concentrations and this was more generalised in the non-amended soils.

Figure 9.11 presents the leaf Cd and Zn concentrations at the different sampling periods and figure 9.12 presents the peduncle and flower concentrations. Cd and Zn concentrations varied according to both the plant tissue and the phenological stage. Shoot Pb concentrations were consistently low in all plant tissues (<20 mg kg^{-1} in non-amended soil and <10 mg kg^{-1} in compost-amended soil). In non-amended soils, the highest Cd concentrations were found in the leaves of *N. caerulescens* during the early to mid-flowering stage (mean concentrations ranged from 978 to 1350 mg kg^{-1}). Elevated Cd concentrations were also found in the flowers, reaching maximum values close to those obtained in leaves (mean concentration of 1075 mg Cd kg^{-1} at mid-flowering stage). The lowest Cd concentrations were consistently found in peduncles, which presented Cd concentrations approximately 3-fold lower than leaf concentrations. The highest Zn concentrations were also found in the leaves, and once again, concentrations were highest in the early to mid-flowering stage. Leaf Zn concentrations varied from 7784 (before flowering) to 22052 mg kg^{-1} (at mid-flowering in June 2013). In this case Zn concentrations were significantly higher in peduncles than in flowers (mean concentration of 4463 mg Zn kg^{-1} in peduncles and 3284 mg Zn kg^{-1} in flowers), but both these tissues presented concentrations approximately 2 to 3-fold lower than in leaves. In compost-amended soil, similar trends were found in tissue concentrations of Zn: maximal leaf concentrations were observed at the mid-flowering stage. While for Cd, the highest concentrations were observed over after flowering (mean concentration of 323-379 mg Cd kg^{-1}). In this case, both Cd and Zn concentrations in peduncles and flowers were significantly lower than corresponding concentrations obtained in leaves. Compost addition led to a significant reduction in both Cd and Zn concentrations, and this was observed in all three tissues. Compost-induced reductions in metal concentrations were more pronounced in the case of Cd: during the early- to mid-flowering stage leaf Cd concentrations were reduced by up to 8-fold (compared to 2.7-fold in the case of Zn over the same period).

As observed for macro- and micro- nutrients, the intercropping of *N. caerulescens* with *L. corniculatus* led to a significant increase in shoot metal concentrations, and this effect was observed in both non-amended and compost-amended soil (albeit not always significant). At harvest (after 1.5 years growth) both leaf Cd and Zn concentrations were significantly higher in *N. caerulescens*

when this plant was intercropped than when it was grown in monoculture. Leaf Cd concentrations were 1.1- to 1.4-fold higher intercropped plants grown in non-amended or compost-amended soils. Intercropping also led to a significant increase in both the Cd (only in compost-amended soil) and Zn BCF ($p < 0.05$) (Figure 9.9). At harvest the mean Cd BCF in *N. caerulescens* grown in compost-amended soil and in monoculture was 107 and this increased to 152 when grown in intercropping. Similarly, Zn BCF values of 75 and 64 were obtained for plants grown in monoculture and of 110 and 93 when grown in intercropping in non-amended and compost-amended soil, respectively. Leaf Zn concentrations were 1.2- to 1.3-fold higher in intercropped plants grown in non-amended or compost-amended soils (Figure 9.11). Moreover, the increase in leaf Zn concentrations was observed throughout the full flowering period in plants grown in compost-amended soil (leaf Zn concentrations were 1.6- to 2.5-fold higher in these plants over this period).

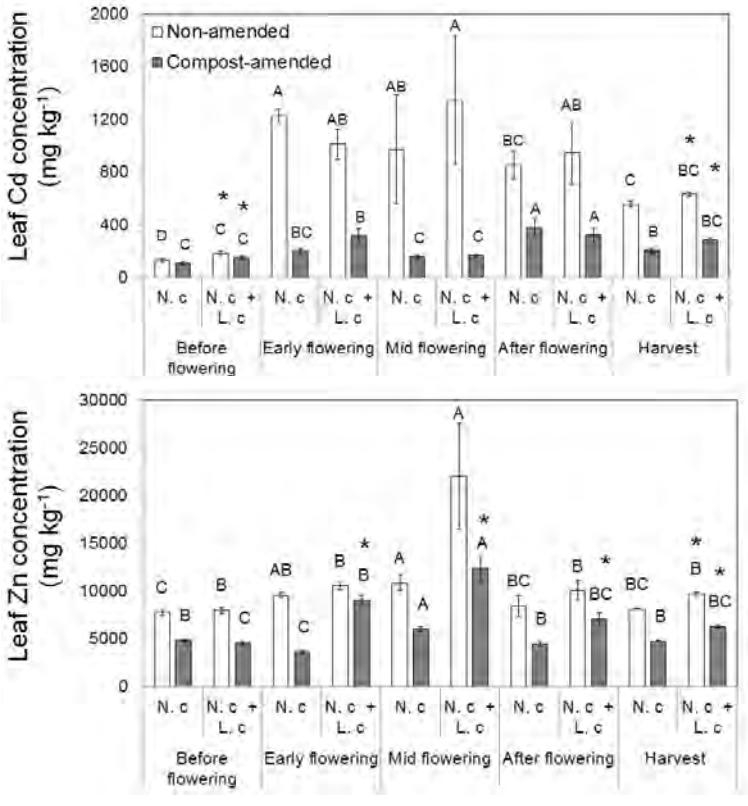


Figure 9.11 Leaf Cd and Zn concentrations of *Noccaea caerulescens* growing in non-amended soils and compost-amended soils. Different capital letters indicate differences between sampling times and asterisks denote significant differences between monoculture and intercropping with *Lotus corniculatus* ($p < 0.05$). N.c, *Noccaea caerulescens*; N.c + L.c, *N. caerulescens* + *L. corniculatus*.

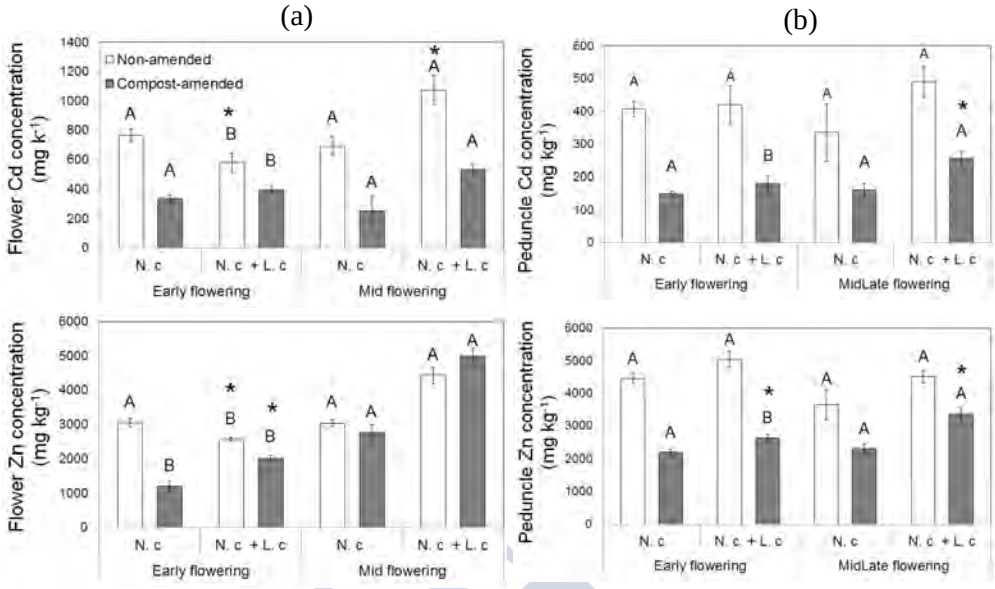


Figure 9.12. Flower Cd and Zn concentrations in flowers (a) and peduncle (b) of *Noccaea caerulescens* growing in non-amended soils and compost-amended soils. Different capital letters indicate differences between sampling times and asterisks denote significant differences between monoculture and intercropping with *Lotus corniculatus* ($p < 0.05$).N.c, *Noccaea caerulescens*; N.c + L.c, *N. caerulescens* + *L. corniculatus.*

Similarly, intercropping had a significant effect on Cd/Zn concentrations in peduncles/flowers: at the mid-flowering stage an increase in the concentration of these metals was consistently observed in both peduncles and flowers (Figure 9.12).

Finally, Cd and Zn yields obtained after harvesting differed significantly between non-amended and compost-amended soil, and in the former they also differed between cropping patterns (Table 9.4). Zn yields were significantly higher than Cd yields, and this was more pronounced in compost-amended soil (Zn yield was up 22- to 23-fold higher than Cd yield) than non-amended soil (Zn yield was 7- to 15-fold higher than Cd yield). In agreement with plant DW biomass, the total phytoextracted Zn (Zn yield) was significantly higher in compost-amended soil than non-amended soil (1.7- to 2.8-fold higher in monoculture or intercropping, respectively). In contrast, compost addition had no effect on the final Cd yield. In the non-amended soils, the intercropping of *N. caerulescens* with the leguminous species had a negative effect on both Cd and Zn yield, however in the compost-amended soil no significant effect of intercropping was observed on the phytoextracted metal.

9.4. Discussion

The mine tailings in the area where the field experiment was implemented were either bare of vegetation (RP experimental block), or showed a sparse vegetation primarily colonised by grassy species (R block). The tailings soils presented a large degree of heterogeneity but generally showed unfavourable conditions for plant growth: low organic matter content, lack of nutrients and nutrient imbalance (Ca dominated CEC (>95%)), and elevated pseudo-total and bioavailable concentrations of some trace metals. Non-amended soils were deficient in essential nutrients, such as N, P, K and Mg. Exchangeable K concentrations were below the threshold level which is considered to indicate deficiency in this element (i.e. <0.2 $cmol_c\ kg^{-1}$ in the exchange complex), while both Mg and P were close to these threshold levels (0.4 $cmol_c$ Mg kg^{-1} (Buol *et al.*, 1975) and $NaHCO_3$-extractable concentration of 5 mg P kg^{-1} (Olsen *et al.*, 1954)). Moreover, the soils presented severe nutrient imbalance: K represented <1% of the base cation saturation, and the Ca:Mg ratio ranged from 24 to 30 (which is close to the Ca:Mg ratio of 50 considered to reflect a strong imbalance between these two elements; (Buol *et al.*, 1975).

The principal metal contaminants in the mine tailings are Cd, Pb and Zn, and both total and bioavailable concentrations showed a high level of heterogeneity as is characteristic of many mine tailings (Barrutia *et al.*, 2011). The presence of these three metals is in accordance with the mineralogy of the orebody which is mainly represented by sphalerite and galena. Cd has also been found in some varieties of sphalerite at this site (Arias-Prieto, 1991). Monterroso *et al.* (2014) classified the soils across these mine tailings on the basis of their total metal concentrations into four categories. Total metal concentrations were compared with the generic reference levels (NGR in Spanish) established for soils in Galicia developed over limestone (Macías and Calvo de Anta, 2009), with phytotoxicity threshold levels (Kabata-Pendias, 2010) and with reference levels for soils under distinct land uses (soil screening levels; EPA (2003)). Accordingly, soils were classified into four categories: I) background (concentrations lower than the NGR values), II) tolerable (concentrations between background levels and phytotoxic levels), III) non-tolerable (concentrations above the phytotoxic thresholds), and IV) highly contaminated (concentrations above those stipulated by the soil screening levels for industrial soils). These authors found that the Zn levels of the mine tailings soils in this site always fell into categories III and IV (non-tolerable to highly contaminated), and the Pb and Cd levels were generally classified as non

-tolerable (category III).

The residual fraction was dominant for Pb, however, both Cd and Zn were amply represented by potentially labile fractions (particularly the water-soluble, exchangeable and acid-extractable). The sequential fractionation is in agreement with previous studies showing the importance of carbonates (up to 10%) in these mine-soils (Becerra-Castro *et al.*, 2012). Monterroso *et al.* (2014) suggested that the high proportion of carbonate-bound metals could be a consequence of the selective extraction of sulphides during ore processing, which would result in carbonate-rich residues, or the result of posterior processes and a re-distribution of metals. During the flotation process of sulphides and dumping of the resulting slurries, the generation of anoxic conditions would favour the solubilisation of Fe/Mn oxides and their associated metals. In addition, oxidising conditions generated at the surface of the tailing ponds during re-vegetation could lead to the liberation of metals from residual sulphides and their posterior re-precipitation in more labile phases, such as carbonates or soluble salts. Minerals such as smithsonite ($ZnCO_3$), cerussite ($PbCO_3$) or malachite ($Cu_2CO_3(OH)_2$) have been found as a result of the supergene alteration of sulphides (Luque, 1985). Environmental conditions or plant root activity inducing important changes in redox potential (oxidation or reduction) or acidification could lead to the liberation of these metals into their surroundings.

Improvements in soil properties and promotion of plant biomass by compost addition are widely described in metal-contaminated soils (Alvarenga *et al.*, 2008; Alburquerque *et al.*, 2011; Pardo *et al.*, 2013). In the present study, amending with compost had a strong and significant effect on both soil fertility and metal availability and fractionation. N, P, K and Mg were no longer present at deficiency levels (increasing by 3 to 24-fold, 7 to 20-fold, 9 to 33-fold and 5 to 7-fold, respectively), and the addition of compost helped improve the strong nutrient imbalance present in the non-amended soils. The mean Ca:Mg ratio in compost-amended soils ranged from 2 to 5 compared to 23 to 30 in non-amended soils. Compost addition did not always lead to an increase in CEC (as expected), and in RPC it significantly decreased CEC. This reduction was possibly due to the occlusion of exchanges sites by added organic matter. Compost addition also led to a significant decrease in soil pH, which could be related to the mineralisation of organic matter after its incorporation into soil. Alongside these changes, a decrease in the availability of Zn, Cd and Pb of approximately 2-fold was observed. Organic wastes can reduce the solubility and availability of metals in

contaminated soils, mainly due to their alkaline character and the complexing capacity of OM (Mench *et al.*, 2003; de la Fuente *et al.*, 2011; Alvarenga *et al.*, 2014). Composted organic residues present a high proportion of humified OM which decreases metal availability through adsorption and the formation of stable organo-metal complexes (Clemente and Bernal, 2006). In addition, cationic metals bind to exchange sites on the OM surface which reduces their mobility (Soler-Rovira *et al.*, 2010). The presence of other compounds in the compost such as phosphates or Fe/Al (hydro)oxides can also lead to the precipitation of metals through the formation of insoluble metal complexes (Brown *et al.*, 1998). Organic amendments can also lead to a re-distribution in trace metal fractionation from soluble and exchangeable forms to pools associated with OM, iron oxides or the residual fraction (Clemente and Bernal, 2006; Alvarenga *et al.*, 2009; Alburquerque *et al.*, 2011; Kumpiene *et al.*, 2011). In this study, there was a relative decrease in the more available fractions of all three metals and increase in less available fractions (such as those bound to OM and sulphates or the residual fraction). Finally, significant effects were observed in soil enzyme activities after compost amendment. Hydrolase activities are related to the mineralisation of nutrients such as C (arylesterase, invertase and β-glucosidase), N (urease), P (phosphomononesterase) and S (arylsulphatase) and are considered to be relevant indicators of soil quality (Gil-Sotres *et al.*, 2005). On the other hand, oxidoreductases (catalase and dehydrogenase) are considered indicators of general metabolic microbial activity (Leirós *et al.*, 2000). Compost addition led to a significant increase in soil enzymatic activity of all evaluated enzymes, except arylsulphatase (only in block RP) and catalase (which was even reduced). The latter could be due to the presence of an established bacterial community in the study area (bearing in mind that the site was not always completely bare of vegetation before the experiment was initiated) which is presumably adapted to the edaphic-climatic conditions and was possibly negatively affected by the addition of the amendment. Parallel studies which were carried out at this site using the denaturing gradient gel electrophoresis (DGGE) technique showed that compost application induced important shifts in the bacterial community structure of the mine tailings (unpublished results).

Two vegetation covers were effectively established, based on either the SRC system using *Salix* spp. or the Cd/Zn hyperaccumulator *N. caerulescens*. Heavily contaminated mine tailings are generally considered unsuitable for the cultivation of food or fodder crops but the production of energy crops, such as the SRC of

willow evaluated in this study, can offer a sustainable method for the phytomanagement of this type of marginal land (Schröder *et al.*, 2008; Ruttens *et al.*, 2011). Alternatively, cultivating metal hyperaccumulators in trace metal-contaminated mine tailings has been recently proposed as a sustainable means of producing metal-enriched biomass for application in Green Chemistry (Losfeld *et al.*, 2014). Metallic cations derived from the biomass of these hyperaccumulator plants can be directly used as Lewis acid catalysts for organic chemistry (Losfeld *et al.*, 2012; Losfeld *et al.*, 2014).

Metal phytotoxicity together with other abiotic and biotic stress factors (water stress, nutrient deficiency, adverse soil pH, soil compaction, herbivory, pests etc.) can severely limit the performance and establishment of the plant species used in phytomanagement (Petcu *et al.*, 2001; Kidd *et al.*, 2015). The careful selection of plant species and optimisation of growth are therefore key elements in successful phytoremediation of trace element-contaminated soils. A large number of *Salix* and *Populus* clones have been screened, and show great variation in biomass production, trace element tolerance and accumulation patterns in roots and leaves between clones (Landberg and Greger, 1994; Pulford *et al.*, 2002; Migeon *et al.*, 2009; Ruttens *et al.*, 2011; Van Slycken *et al.*, 2013b). Here, two *Salix* species were cultivated; however, since the plots have not yet been harvested it was not possible to evaluate their biomass production. In terms of growth, both species reached their maximum height after 2 years of growth. The best-performing species (in both non-amended and compost-amended soil) was *S. smithiana*, with plants reaching up to 1.9 m in height and mortality lower than 30%. In contrast, the maximum height reached by *S. atrocinerea* was 1.1 m and in compost-amended soils mortality was 100% (in both monoculture and intercroppings). In addition, *S. atrocinerea* was not able to grow in combination with *A. glutinosa* in non-amended soils, and mortality was close to 100% after 3 years of growth. This was most likely due to competition effects between the young willows and weeds (particularly prevalent in compost-amended soils) or in intercropped plots with the *A. glutinos*a (which was fast-growing and reached a similar size to that of *S. smithiana*). Compost-amended soils were rapidly colonised by a high density of weeds, thus reinforcing the need for weed control at least during the initial plant establishment phase (Kidd *et al.*, 2015). In contrast, mortality of *N. caerulescens* was low in both non-amended and compost-amended soils (< 30%). Moreover, in the compost-amended soils the biomass production was up to 3-fold higher than in non-amended plots. In this case the high densities

of plants could have contributed towards reducing the germination of weeds and limiting their proliferation.

Plant growth had a significant effect on soil physico- and bio-chemical properties, and these effects were dependent on the plant species and cropping pattern. Growth of both *S. smithiana* and *S. atrocinerea* induced a significant decrease in soil pH in non-amended soils, but this was only statistically significant in NPK-fertilised soils. Both species and cropping patterns were also shown to significantly increase the NH_4Cl-extractable concentration of Cd and Zn, and this was observed in both non-amended and compost-amended soils. This effect on metal availability was more pronounced in the NPK-fertilised plots. Since N was applied as NH_4NO_3 the decrease in soil pH may have been a consequence of soil nitrification. Ammonium fertilisers frequently cause higher metal concentrations in crops than nitrate fertilisers as a result of the pH decrease caused by nitrification or plant uptake of NH_4^+ (Grant *et al.*, 1999). On the other hand, plant availability of metals can be affected directly through the addition of the metal as a contaminant in the P fertiliser (i.e. Cd), or indirectly through ion exchange reactions in the soil solution (McLaughlin and Singh, 1999; Wångstrand *et al.*, 2007). The enhanced Cd availability in the NPK-fertilised treatment could also be related to the form of K used (KCl). Chloride-based fertilisers have been shown to influence soil Cd mobility through the formation of chloride complexes (Smolders *et al.*, 1998), and this is particularly pronounced in alkaline soils (Hattori *et al.*, 2006). The results obtained here show that the chemical form of fertilisers can be selected and managed according to the GRO technique being implemented. In this study, the target was Cd/Zn phytoextraction and ammonium- and chloride-based fertilisers were chosen as a means of enhancing the phytoavailability of the metallic cations. However, as will be discussed further below, corresponding increases in plant metal uptake were not obtained despite the increase in metal availability. The plant-induced increase in metal availability was less pronounced in the compost-amended soils (under both monoculture and intercropping treatments with *S. smithiana*).

Growth of *N. caerulescens* (in both cropping patterns) was also found to decrease soil pH, but in this case plant-induced acidification was only observed in compost-amended soil (where growth was more prolific). In contrast, growing the hyperaccumulator led to a significant reduction in CEC in non-amended soils, which was largely attributed to a reduction in exchangeable Ca. In both non-amended and compost-amended soils the presence of the hyperaccumulator

tended to reduce the NH_4Cl-extractable concentrations of Cd (except for intercropping treatments in compost-amended soil) and significantly reduced Zn concentrations. The reduction in NH_4Cl-extractable Zn concentrations was again more pronounced when plant growth was greater, and was most likely due to plant uptake and accumulation. Epelde *et al.* (2008) also showed that the growth of *N. caerulescens* induced changes in soil properties of artificially metal-contaminated soils: plant growth decreased both available P and CEC, increased soil pH, and decreased the phytoavailable fraction ($CaCl_2$-extractable concentration) of Zn and Pb (by 14% and 27%, respectively). In this study, NH_4Cl-extractable concentrations of Pb were not significantly influenced.

The presence of both plant species led to a significant and progressive increase in the activities of most assayed soil enzymes compared to non-vegetated soils. This may be due to the amelioration of soil physicochemical properties caused by the establishment of the willow and *Noccaea*, which are likely to have improved soil physical properties (structure, aeration, water retention) and nutrient availability (increased by root exudates in the rhizosphere). Additional causes could be the release of enzymes by the plant roots, enhanced microbial enzyme synthesis in the rhizosphere (Grierson and Adams, 2000; George *et al.*, 2005), and/ or the protective effect on extracellular enzymes by soil OM (Burns, 1982). In the case of the hyperaccumulator, the stimulation in soil enzymatic activities may also be related to the decrease in soil Cd/Zn availability and hence toxicity. This has been previously shown in other studies carried out in trace metal-contaminated sites (Epelde *et al.*, 2008; Renella *et al.*, 2008; Kumpiene *et al.*, 2009). Xue *et al.* (2015) evaluated the effects of phytoextraction on soil health in a site located in Lommel (Belgium) which was diffusely contaminated with Pb, Cd and Zn due to historic smelter activities. The growth of the metal-accumulating willow clone 'Tora' (*Salix schwerinii* x *Salix viminalis*) increased soil microbial biomass and respiration, functional gene richness and diversity, and soil enzymatic activities to a greater extent than control plots under grassland. Epelde *et al.* (2008) also found that the growth of *N. caerulescens* stimulated the activities of urease, ß-glucosidase, arylsulphatase and acid and alkaline phosphatase compared to unplanted soils. As a consequence of the metal phytoextraction process the activity of these five soil enzymes increased, which have a key function in soil nutrient cycling. In the present study, the intercropping of the *S. smithiana* with *A. glutinosa* also had a more pronounced positive effect on soil enzymatic activities (particularly urease and alkaline phophomonoesterase) than when grown in mono-

culture. A similar effect was observed with the hyperaccumulator, where soils under intercropping showed higher activities in urease and phosphomonoesterases (although only in the first growth season) than under monoculture. A synergistic effect was also observed between the compost amendment and plant cropping system, with a greater stimulation in soil enzyme activities under intercroppings in compost-amended soils. These results therefore confirm that the presence of these legumes within the phytoextraction system plays an active role in N and P cycling and availability. The DGGE technique carried out in parallel studies also demonstrated shifts in the bacterial community between unplanted and planted soils, and the presence of communities associated with either woody tree species or the hyperaccumulator. Bacterial diversity indices based on the DGGE fingerprints also indicated a higher diversity in planted soils than unplanted soils. Moreover, differences in the bacterial community structure were observed between monoculture or intercropping patterns (unpublished results).

The concentrations of Cd and Zn in the mine-soils were higher than the threshold values considered toxic for normal plants, and plant tissue concentrations of these two elements exceeded the critical values of phytotoxicity (Kabata-Pendias, 2010). A large variation in metal accumulation by different *Salix* species and/or clones is frequently found in the literature. Unterbrunner *et al.* (2007) found concentrations of up to 166 mg Cd kg^{-1} or 438 mg Pb kg^{-1}, and between 1000 and 3000 mg Zn kg^{-1}, in leaves of adult trees of *S. caprea* growing in long-term Cd- and Zn-contaminated areas across Central Europe. Dos Santos Utmazian and Wenzel (2007) described the *Salix smithiana* clone (BOKU 03 CZ-001) as having an excellent capacity to accumulate Cd and Zn in leaves (up to 400 mg Cd kg^{-1} and 2000 mg Zn kg^{-1}). The same clone was evaluated by Puschenreiter *et al.* (2013), who found leaf concentrations of up to 35.3 - 96.4 mg Cd kg^{-1} and 423 - 2670 mg Zn kg^{-1} after two years of cultivation in Cd/Zn-contaminated agricultural soil. The corresponding phytoextracted metal (relative to the total concentration) was up to 10% for Cd and between 1.2-2.5% for Zn. However, neither of these studies was carried out at a field-scale. The results of this field trial confirm that this *S. smithiana* clone is an efficient Cd/Zn accumulator, reaching Zn concentrations in the leaves of more than 4000 mg kg^{-1} after 1 year's growth and up to 21 mg kg^{-1} Cd after 3 years.

S. atrocinerea accumulated similar concentrations of Cd as found in *S. smithiana*, while Zn concentrations were approximately 2-fold lower (up to 28 mg Cd kg^{-1} and 2000 mg Zn kg^{-1}, respectively). However, these concentrations were

markedly higher than concentrations reported for these two metals in *S. atrocinerea* growing spontaneously at the same mine area (Becerra-Castro *et al.* (2012); Zn concentrations ranged from 300 to 900 mg kg^{-1}). They are also higher than those found in shoots of the same clone growing spontaneously in an abandoned pyrite mine close to Madrid (Moreno-Jiménez *et al.*, 2009).

Unfortunately, no significant reductions in the total concentrations of Cd or Zn were found under either *Salix* spp. This may be due to the fact that a longer time frame is needed before significant reductions in total metal concentrations are seen. Moreover, NH_4Cl-extractable concentrations of Cd and Zn were higher in soils under *Salix* spp. compared to unplanted soils. This is probably due to root activity and plant-induced changes in soil physicochemical properties, but could also be a result of the re-incorporation of metal-enriched leaf litter fall into the soil. Nonetheless, given its greater growth and higher capacity to accumulate Zn, the *S. smithiana* clone (BOKU 03 CZ-001) would be a better candidate for phytoextraction of this metal.

Metal accumulation in *N. caerulescens* was well above the threshold for Cd and Zn hyperaccumulation (100 mg Cd kg^{-1} and 10000 mg Zn kg^{-1}). No signs of phytotoxicity were found in either non-amended or compost-amended soils. Phytoextraction of Cd and Zn with the hyperaccumulator *N. caerulescens* has been well documented (Lombi *et al.*, 2000; Hammer *et al.*, 2003; Zhao *et al.*, 2003; Yanai *et al.*, 2006). However, few studies have been carried out at a field-scale. This species has been found to accumulate up to 10000 mg Cd kg^{-1} and 30000 mg Zn kg^{-1} in its aerial parts without showing negative effects when grown in sewage sludge-amended agricultural soils (Brown *et al.*, 1995). Metal accumulation by the hyperaccumulator in the present study was of a similar magnitude for Zn but lower for Cd: shoot concentrations were up to 32000 and 17000 mg Zn kg^{-1}, and 2300 and 480 mg Cd kg^{-1}, in the non-amended and compost-amended soils, respectively. A field experiment carried out by Schwartz *et al.* (2003) evaluated the efficiency of *N. caerulescens* to phytoextract Cd and Zn over a 5 year period from both an agricultural soil amended with trace metal-rich urban sewage sludge and an industrial soil co-contaminated with metals and organic compounds (from a former coking plant). Plants accumulated up to 40 mg Cd kg^{-1} and two successive crops of the hyperaccumulator extracted up to 9% of the total Cd and 7% of the total Zn. The available Cd/Zn fraction (NH_4NO_3-extractable concentration) was also significantly decreased. A more recent field study by Simmons *et al.* (2015) evaluated the potential of *N. caerulescens* populations (from southern France) to

phytoextract localised Cd/Zn contamination in rice paddy soils in Thailand. Compared to the present study, shoot Cd concentrations presented a similar range (460–600 mg kg^{-1} DW) to our results; while shoot Zn concentrations were up to one magnitude lower (2600–2900 mg Zn kg^{-1} DW). Simmons *et al.* (2015) reported Cd and Zn bioaccumulation factors ranging from 12–15 and 1.2–1.3, which were one to two orders of magnitude lower than those obtained here. Here, after one crop of *N. caerulescens* the available Zn concentration was reduced by up to 1.5- and 2.7-fold in non-amended and compost-amended soils, respectively. The hyperaccumulator was therefore efficient in reducing the labile Zn pool in the upper soil layer (0-20 cm), however, for phytoextraction of deeper soil layers this species would most likely have to be combined with a deeper rooting metal-(hyper)accumulating plant species e.g. *Salix* spp.

Metal accumulation was strongly compartmentalised in both *Salix* spp. and *N. caerulescens* and primarily occurred in the leaves (except for Cd which did not differ between leaves and stems in the *Salix* spp). Higher concentrations of Cd and Zn in the leaves of Salicaceae have commonly been reported (Pulford and Watson, 2003; Janssen *et al.*, 2015; Kidd *et al.*, 2015). Moreover, the leaf biomass production of willows has been shown to represent a significant part of their yearly total biomass production. Ruttens *et al.* (2011) found leaf biomass production of willow ranged between 1468 kg and 2633 t ha^{-1} yr^{-1}, representing 18% to 40% of the mean yearly total biomass production of these clones. Vyslouzilova *et al.* (2003) showed that the larger part of phytoextracted Cd and Zn corresponded with willow leaves (83% and 71% of the total extracted Zn and Cd). At the Lommel site (Belgium) between 81 to 121 g Cd ha^{-1} yr^{-1} could be removed by poplars when harvesting only twigs and up to 187 to 240 g Cd ha^{-1} yr^{-1} if leaves were also harvested (Vangronsveld *et al.*, 2009). It is therefore important to consider harvesting of leaves in SRC phytoextraction systems (Dickinson and Pulford, 2005; Hammer *et al.*, 2003).

Metal accumulation by *N. caerulescens* varied according to the plant phenological stage, confirming that the optimal harvesting time should be carried out in line with both biomass production and metal accumulation. Here, maximal shoot Cd/Zn concentrations were found at the mid- to late-flowering stage. A similar trend was observed by (Bani *et al.*, 2015a) in the Ni-hyperaccumulator, *Alyssum murale*, when cultivated in the ultramafic area of Pojskë (Albania). These authors found that the best harvesting time with maximum biomass production and Ni accumulation was the mid-flowering stage. Harvesting at this time also avoids

the formation of seeds and the introduction of these plants in areas where they are not native to the local flora. The high concentrations of Cd and Zn found in the flowering shoots (peduncles) and flowers (up to 1075 mg Cd kg^{-1} and 5055 mg Zn kg^{-1}) would only enhance the overall metal removal during the phytoextraction process.

Two strategies were evaluated as a means of improving the process of Cd/ Zn phytoextraction: the addition of organic amendments and the intercropping of the phytoextracting plant species with N_2-fixers. A beneficial effect of compost addition on plant growth was only observed in *N. caerulescens*. Both plant species showed P, K, Ni or Co deficiency (according to threshold levels given by Marschner (1995)) when growing in the non-amended soils. Despite the strong nutritional limitations the hyperaccumulating species was more efficient at obtaining essential nutrients (particularly K) than the woody crop species. The hyperaccumulator was also more efficient at increasing exchangeable K in its surrounding soil and improving the strong nutritional disequilibrium (e.g. the Ca/ Mg ratio). However, compost addition was able to significantly improve the nutritional status of both *Salix* spp. and *N. caerulescens*, and shoot nutrient concentrations were above threshold limits of deficiency in this treatment.

The better plant performance, alongside the improvements in soil physico- and bio-chemical properties, supports the use of these amendments in this type of phytomanagement. However, an important factor that must be taken into careful consideration is the significant reductions in shoot Cd and Zn concentrations observed in all species when grown in compost-amended soils compared to non-amended soils, which are in line with the reduction in soil metal availability after compost addition. A decrease in shoot trace metal concentrations after addition of organic amendments to metal-contaminated soils is a common phenomenon and has been observed in numerous plant species, including woody species, agricultural crops, and grass species (He and Singh, 1993; Narwal and Singh, 1998; Walker *et al.*, 2004). Compost addition can clearly increase soil organic matter content and fertility; however, given their effects on metal availability it will be important to optimise the rates of addition when the aim is to phytoextract metals. Further experiments evaluating the effectiveness of split additions or the long-term duration of the effects of compost addition would also be useful. The massive increase in shoot DW yields of the hyperaccumulator obtained using compost amendment compensated for the reduction in shoot Cd and Zn concentrations, so that, at least in the case of Zn, the overall yields were

still greater in compost-amended plots compared to non-amended plots: the mean Zn yield was 5.9 kg ha^{-1} in non-amended soil compared to 10 kg ha^{-1} in compost-amended soil, while Cd yields varied from 0.41 to 0.43 kg ha^{-1}.

Cropping patterns which include leguminous species have also been proposed as a means of improving the productivity of phytoextracting plant species; although few field evaluations can be found (Kidd *et al.*, 2015). The positive below-ground, plant-soil and plant-plant interactions can result in an improved nutrient availability and/or increased crop yield (through N_2 fixation, transfer of fixed N and mobilisation of P due to rhizosphere acidification). Frérot *et al.* (2006) found perennial grass mixtures which included the legume, *Anthyllis vulneraria*, achieved a higher coverage, yield and improved nutrient status in highly polluted soils (with Cd/Zn/Pb) compared to field plots with only grasses. In the present study intercropping of *S. smithiana* with *A. glutinosa* only had a beneficial effect on growth in compost-amended plots, and no effects were observed on plant nutrition in either non-amended or compost-amended soils. Similarly, no beneficial effects of NPK fertilisation were found on the nutritive status of the tested *Salix* spp. (despite the enhanced growth). Thus, neither the use of inorganic fertilisation nor intercropping with legumes was able to consistently improve growth and nutrition of the woody crops. It is possible that a longer time frame is required to observe a beneficial effect of these two treatments on plant productivity and health in this species. In contrast, after 3 years growth an effect of plant cropping patterns was observed in Cd/Zn accumulation in the stems of *S. smithiana*, where stem concentrations of both metals were higher in intercropped plants than monocultures. Moreover, BCF values for both metals were significantly higher in this intercropping treatment. It is possible that, in the long-term, beneficial effects of intercropping may be seen on the metal phytoextraction capacity of *S. smithiana*.

When *N. caerulescens* was intercropped with *L. corniculatus* its growth was actually decreased but only in the non-amended soil. In contrast, beneficial effects were always observed in shoot nutritive status, and were generally more pronounced in the compost-amended soils. In addition, shoot Cd and Zn concentrations, and both Cd and Zn BCF values, were significantly greater in *N. caerulescens* intercropped with *L. corniculatus* compared to *N. caerulescens* in monoculture. The lower Cd and Zn yields obtained when *N. caerulescens* was intercropped with the legume in non-amended soils were therefore due to the lower plant biomass in this soil (together with the lower planting density in inter-

cropped plots). Further studies optimising the plant densities of both the hyperaccumulator and the legume could reduce or avoid these negative effects on growth, especially if these were a result of competition for space and/or resources. For example, in the compost-amended soil the larger size and competitive ability of the hyperaccumulator in this soil did not lead to any significant reduction in plant growth, and the lower metal yields were simply due to the lower planting density in intercropped plots. Arco-Lázaro *et al.* (2015) observed that intercropping the high biomass crop *Piptatherum miliaceuem* with the legume *Bituminaria bituminosa* increased growth of the high-biomass crop. Moreover, these authors obtained best results in terms of biomass productivity when combining compost amendment and intercropping with the legume. Wieshammer *et al.* (2007) found that intercropping the two metal-accumulating species, *Salix caprea* and *Arabidopsis halleri*, led to reduced shoot biomass and metal concentrations of both species due to competition for metal uptake and possibly also nutrients and water. Bani *et al.* (2015b) showed that the hyperaccumulator *A. murale*, at intermediate plant densities, was able to produce a higher biomass (and therefore higher Ni removal from soils) than at higher plant densities. Liu *et al.* (2013) also obtained a lower biomass in *Zea mays* when intercropped with legumes compared to monoculture, and they attributed this to the high densities of the plant species and consequent competition for nutrient resources. Finding the ideal plant density of intercroppings will therefore be a critical factor for obtaining maximal plant shoot yields and metal removal, while capitalising on the beneficial effects on soil fertility and biological activity, plant nutrition and shoot metal accumulation.

9.5. Conclusions

This field study demonstrates the potential of SRC options (particularly using *S. smithiana*) and hyperaccumulator plants (*N. caerulescens*) for the re-vegetation of a moderately Cd/Pb/Zn-contaminated tailings soil, in combination with metal phytoextraction. In addition, the results show that the incorporation of low grade composts (based on agricultural and industrial wastes) can be successfully incorporated into the phytoextraction system, as well as contributing towards the recycling of residues, thus supporting current European policies aimed at reducing wastes (EU's Zero Waste Policy, 2013). Plant cover and compost addition led to improvements in soil physicochemical properties, fertility and biological activity. Compost amendment significantly improved plant

nutritive status, and growth and Cd/Zn removal (the latter only in the case of *N. caerulescens*). A longer time frame may be necessary before the effects of compost amendments or cropping pattern are seen in the SRC, which also reinforces the need for medium- to long-term field experiments before full scale deployment of these techniques can take place. One crop of the hyperaccumulator efficiently reduced soil Zn availability. Depending on the plant species and soil amendment, intercropping phytoextracting species with leguminous plants could improve plant growth and metal uptake and accumulation. Further studies are required to fully optimise the system. Moreover, it would be interesting to evaluate alternative practises, such as bioaugmentation with beneficial plant-associated microorganisms, to improve plant growth and metal phytoextraction in these sites.

9.6. References

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FINAL SYNTHESIS AND CONCLUSIONS

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The characterisation of the culturable bacterial community associated with plant species from serpentine and non-serpentine soils showed that bacterial strains isolated from the serpentine soils present a higher resistance to Ni than those from the calcareous soil. Moreover, the Ni-hyperaccumulators (both *Alyssum pintodasilvae* and *A. malacitanum*) hosted a higher density of Ni-resistant bacteria in their rhizosphere than either non-accumulating plants (*Dactylis glomerata* and *Santolina semidentata*) or non-vegetated soil. The highest densities of Ni-resistant bacteria were found in the rhizosphere to *A. pintodasilvae* from Morais and *A. malacitanum* from S. Bermeja, and this was related to the higher water-soluble Ni concentrations observed in their rhizosphere soils. A total of 550 strains were isolated and characterised. Half of these isolates showed at least one plant growth promoting (PGP) trait. A substantial number were able to produce organic acids, siderophores or indoleacetic acid (IAA), while the rarest phenotypes were the biosurfactant production and the ability to solubilise inorganic phosphate. The culturable rhizobacterial communities were dominated by the phyla *Proteobacteria* and, in particular, *Actinobacteria* and isolates were identified as members of genera such as *Arthrobacter*, *Streptomyces*, *Rhodococcus* or *Microbacterium*. A small number of strains, only found in association with the *Alyssum* hyperaccumulators, were identified as interesting candidates for further studies related to the application of Ni-hyperaccumulating species in phytomining.

When evaluating the soil Ni availability in the rhizosphere of *A. pintodasilvae* and *Holcus lanatus* both plant species were found to influence soil physicochemical characteristics. However, plant-induced changes in Ni availability and fractionation were more pronounced in the case of the hyperaccumulator. The DGT technique was a more sensitive tool for highlighting these plant-induced changes than the conventional extractions. The results suggest that the enhanced Ni availability in the rhizosphere of *A. pintodasilvae* could be a consequential effect in response to the plant's efforts to improve its nutrition through mineral weathering and/or mineralisation of organic matter. However, more studies are required to characterise the composition of the root exudates released by both plant species and further unravel the role that dissolved organic carbon (DOC) plays in determining soil Ni availability.

Soil inorganic fertilisation (NPK) and soil organic amendments increased the total amount of Ni phytoextracted by four Ni-hyperaccumulator (sub)species (*A. pintodasilvae*, *A. malacitanum*, *A. bertolonii* and *Noccaea goesingensis*) and this was mainly through a stimulation in biomass production (up to 162-fold

increase in biomass production of *N. goesingensis* after 5% compost addition). The most efficient plants in terms of Ni removal were *N. goesingensis*, *A. bertolonii* and the SB population of *A. malacitanum*. The best soil treatments were the intermediate rates of compost (2.5 or 5.0% w/w) addition (and also the NPK treatment in the case of *N. goesingensis*). The addition of compost to mine soils contaminated with Cd, Pb and Zn significantly increased shoot biomass production of *Nicotiana tabacum* and Zn accumulation in *Salix caprea* growing in these soils. Composts can not only provide a source of essential nutrients (which are limiting in serpentine and metal-contaminated soils) but also improve soil structure and porosity, and water holding capacity, which could explain the more pronounced effects of compost additions that were observed on plant yields compared to inorganic fertilisers. However, strong reductions in soil metal availability (and corresponding plant metal accumulation) were observed after compost addition in both soils, which highlights the need for further optimisation regarding the final rates of addition.

The identification of potential bacterial strains which are able to respond under a range of soil conditions is interesting when transferring bioaugmentation strategies to real-life field scenarios. Few studies have evaluated the effects of bacterial inoculants in combination with soil amendments and using more than one host plant species. Inoculation of *S. caprea* and *N. tabacum* with rhizobacterial strains isolated from a Pb/Zn mine soil showed that the combined effects of soil inoculants (strains P87, P29, P30, P75) together with compost amendments on biomass production of tobacco were more beneficial than when used on their own. Bacterial inoculants effectively improved plant growth, modified soil metal availability, and increased plant metal accumulation. However, the effects of such rhizobacterial strains on plant growth or metal accumulation were highly soil- and plant species-specific. The choice of an appropriate inoculation method also seems to be of crucial importance in the inoculation process, although the optimisation of inoculation methods for plants growing in contaminated soils has rarely been assessed. The effects of the inoculation mode were tested using the IAA-producing bacterial *Rhodococcus erythropolis* (P30 strain) inoculated in tobacco plants. Both the inoculation mode (seed or soil inoculation) and the cellular density of the inoculum were shown to be important factors to take into account when designing inoculation strategies, since they can modify plant performance and soil metal removal. A single soil inoculation event was found to give the best results together with the lower bacterial density (10^6 CFUs mL^{-1}).

The field study carried out at the Touro mine site demonstrated the potential for low grade composts (based on agricultural and industrial wastes) in combination with Cu-excluding plant species for the revegetation of an acidic heavily Cu-contaminated tailings soil. Strong reductions in labile Cu, alongside improvements in physicochemical properties, fertility and biological activity, were maintained over the three year monitoring period. The use of waste-derived amendments in this type of site, such as the compost used here, represent a practical and environmentally sustainable solution for the valorisation of wastes products and is in accordance with sustainable management practices within current European policies. Moreover, the presence of a plant cover stimulated the establishment of biogeochemical cycles in the mine tailings which were previously almost inert.

The field study at the Rubiais mine site evaluating the phytoextraction efficiency of short rotation coppice (SRC) system using *Salix* spp. and the Cd/Zn-hyperaccumulator (*Noccaea caerulescens*) demonstrated that both the fertilisation regime (inorganic NPK fertilisers or amendment with composted organic wastes) and plant cropping pattern (monoculture vs. intercropping with legumes (*Alnus glutinosa* or *Lotus corniculatus*)) can significantly influence soil fertility and health, plant growth and tolerance, nutritive status and metal accumulation. The hyperaccumulator was efficient in reducing labile Zn (and to some extent Cd) concentrations after only one crop, and this was most successful in compost-amended plots where plant growth was greatly enhanced. However, a longer time frame may be necessary before the effects of soil amendments or cropping pattern are seen in the SRC. Results reinforce the need for medium- to long-term field experiments before the final selection of the most adequate remediation technique and its full scale deployment.

The main conclusions of this doctoral thesis can be summarised as follow:

-The isolation and characterisation of Ni-tolerant bacteria from serpentine soils is a valuable means of identifying bacterial strains, as well as plant-bacterial associations, with potential application in phytomining techniques.

-The knowledge of soil processes influencing Ni fractionation at the rhizosphere of the hyperaccumulators is important for the development of strategies focusing on increasing soil metal availability. Soil organic matter and plant nutrition seem to play a fundamental role in determining

soil metal availability in the rhizosphere of *A. pintodasilvae*.

-Agronomic practises such as soil fertilisation or organic amendments and the use of bacterial strains as bioinoculants are useful for the improvement of either phytoextraction (including phytomining) or phytostabilisation techniques.

-Rates of compost addition in phytoextraction experiments should be carefully assessed before their implementation in large-scale field experiments so as to obtain the optimal balance between facilitating revegetation at the same time as minimising reductions in soil metal availability

-The use of industrial by-products as soil amendments can facilitate plant establishment in degraded areas, improve soil properties and fertility, and at the same time represents an environmentally sustainable solution for the valorisation of wastes supporting the current European policies.

-The use of bacterial inoculants can effectively enhance phytoremediation options in combination with soil amendments but their effect is highly plant- and soil-specific. Moreover, the bacterial inoculation method markedly influences the obtained results.

-The field studies carried out demonstrate that phytoremediation (both, phytostabilisation or phytoextraction) options are suitable for the revegetation of contaminated sites, such as mine tailing soils. Moreover, these small-scale experiments are needed for the selection of the best combination of plants and treatments for each specific site before they are fully deployed.

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