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Tesis Doctoral

MICROBIAL INDICATORS FOR THE ASSESSMENT OF THE IMPACT OF METAL CONTAMINATION AND PHYTOREMEDIATION ON SOIL HEALTH

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CONTRIBUCIÓN DEL AUTOR

Yo, Aritz Burges Ruiz, declaro que:

Esta tesis doctoral ha generado cuatro artículos ya publicados en revistas ISI de primer cuartil, así como un artículo actualmente en proceso de publicación:

1. Burges A, Epelde L, Garbisu C, 2015. Impact of repeated single-metal and multi-metal pollution events on soil quality. *Chemosphere* 120, 8-15 (ver Capítulo 4).
2. Burges A, Epelde L, Blanco F, Becerril JM, Garbisu C. Ecosystem services and plant physiological status during endophyte-assisted phytoremediation of metal contaminated soil. *Science of the Total Environment*, doi.org/10.1016/j.scitotenv.2016.12.146 (ver Capítulo 5).
3. Burges A, Epelde L, Benito G, Artetxe U, Becerril JM, Garbisu C, 2016. Enhancement of ecosystem services during endophhyte-assisted aided phytostabilization of metal contaminated mine soil. *Science of the Total Environment* 562: 480-492 (ver Capítulo 6).
4. Epelde L, Burges A, Mijangos I, Garbisu C, 2014. Microbial properties and attributes of ecological relevance for soil quality monitoring during a chemical stabilization field study. *Applied Soil Ecology* 75: 1-12 (ver Capítulo 7).
5. Burges A, Alkorta I, Epelde L, Garbisu C. From phytoremediation of soil contaminants to phytomanagement of ecosystem services in metal contaminated sites. *Enviado para su publicación* (ver Capítulo 1).

La acreditación de los co-autores en los distintos artículos evidencia que el presente trabajo es resultado de una activa colaboración investigadora entre el Grupo de Ecología Microbiana de Suelos de NEIKER-Tecnalia y el Departamento de Biología Vegetal y Ecología de la Universidad del País Vasco (Grupo del Dr. José M. Becerril).

Así mismo, del presente trabajo también son fruto las siguientes comunicaciones a congresos:

1. Burges A, Epelde L., Blanco F, Garbisu C. Searching for microbial indicators of soil stability against repeated heavy metal contamination

events. Eurosoil International Congress 2012, Science for the Benefit of Mankind and Environment. Bari (Italia), 2012.

2. Epelde L, Mijangos I, Martín I, Burges A, Garbisu C. Attributes of ecological relevance for the monitoring of soil quality in a field chemostabilization study. 9th International Phytotechnology Society Conference. Hasselt (Bélgica), 2012.
3. Burges A, del Pino ML, Benito G, Blanco F, Epelde L, Becerril JM, Garbisu C. Characterization of plant growth-promoting bacteria isolated from the rhizosphere of metal tolerant plant species from Pb/Zn mine tailings. V International Conference on Environmental, Industrial and Applied Microbiolgy. Madrid, 2013.
4. Burges A, Benito G, Blanco F, Artetxe U, Epelde L, Becerril JM, Garbisu C. Isolation and characterization of plant growth-promoting endophytic bacteria from pseudometallophytes. International Congress of Phytoremediation of Polluted Soils. Vigo, 2014.
5. Burges A, Benito G, Blanco F, Artetxe U, Becerril JM, Epelde L, Garbisu C. Soil ecosystem services for the evaluation of a metal phytostabilization process assisted with PGPBP. 11th International Phytotechnologies Conference. Heraklion (Grecia), 2014.
6. Artetxe U, Burges A, Barrutia O, Epelde L, Garbisu C, Becerril JM. Efecto de enmiendas orgánicas y/o bacterias promotoras del crecimiento en plantas (PGPB) en poblaciones metalícolas y no metalícolas de *Festuca rubra* creciendo en suelos mineros. NutriPLANTA, XV Simpósio Luso-Espanhol de Nutrição Mineral das Plantas. Lisboa, 2014.
7. Epelde L, Burges A, Benito G, Artetxe U, Becerril JM, Garbisu C. Plant growth-promoting bacteria in metal phytoremediation with *Festuca rubra*, *Noccaea caerulescens* and *Rumex acetosa.* 10th International PGPR Workshop - From Omics to the Field. Lieja (Bélgica), 2015.
8. Burges A, Epelde L, Benito G, Artetxe U, Becerril JM, Garbisu C. Servicios de los ecosistemas para la evaluación de procesos fitorremediadores de metales. CONDEGRES – VII Simposio Nacional sobre el Control de la Degradación y Restauración de Suelos. Bilbao, 2015.

Mi contribución a la realización del presente trabajo es la siguiente:

- La conducción de los experimentos, incluyendo el diseño experimental, así como el tratamiento estadístico de los datos, la discusión de los datos y la redacción de los artículos científicos, en los Capítulos 4, 5 y 6, bajo la supervisión de los Dres. Carlos Garbisu y Lur Epelde, y con la colaboración del Dr. José M. Becerril en los Capítulos 5 y 6.
- La realización de los muestreos, en el Capítulo 7, bajo la supervisión de los Dres. Carlos Garbisu y Lur Epelde.
- La determinación de parámetros físico-químicos y microbianos, en los Capítulos 4, 5, 6 y 7, bajo la supervisión del Dr. Fernando Blanco.
- La puesta a punto y validación de los procedimientos de aislamiento de bacterias endófitas y determinación de características promotoras del crecimiento vegetal, en los Capítulos 5 y 6, bajo la supervisión del Dr. Fernando Blanco y con la colaboración de Garazi Benito.
- La estimación de servicios de los ecosistemas mediante la agrupación de parámetros edáficos, en los Capítulos 5 y 6, bajo la supervisión de los Dres. Carlos Garbisu y Lur Epelde.
- La germinación y crecimiento de las plantas empleadas en los Capítulos 5 y 6, bajo la supervisión del Dr. Unai Artetxe.

Además, para la realización de esta tesis también ha sido necesaria la colaboración de otras personas, cuya contribución quiero reconocer en este apartado, en las siguientes tareas:

- El diseño experimental del Capítulo 7 fue llevado a cabo por el Dr. Iker Mijangos, en colaboración con los Dres. Carlos Garbisu y Lur Epelde.
- El tratamiento estadístico y la discusión de datos del Capítulo 7, así como la redacción del artículo científico, fue llevado a cabo por la Dra. Lur Epelde, bajo la supervisión del Dr. Carlos Garbisu.
- La estimación de atributos de relevancia ecológica, en el Capítulo 7, fue llevada a cabo por la Dra. Lur Epelde, bajo la supervisión del Dr. Carlos Garbisu.
- El análisis de los parámetros fisiológicos de plantas, correspondientes a los Capítulos 5 y 6, fue llevado a cabo por el Dr. Unai Artetxe.

- El análisis de las muestras mediante qPCR, en el Capítulo 5, fue llevado a cabo por el Dr. Iker Martín.
- El análisis ARISA, en los Capítulos 5 y 6, fue llevado a cabo por los Dres. Irati Miguel y Fernando Rendo del Servicio de Secuenciación y Genotipado (SGIker) de la Universidad del País Vasco.

Finalmente, de cara a promover la divulgación del trabajo aquí presentado, se ha realizado un breve vídeo con los aspectos más relevantes de la investigación desarrollada en esta tesis (ver: www.soilmicrobialgroup.com/video-tesis-aritz-burges)

RESUMEN

El suelo es un sistema dinámico y complejo, cuyas funciones son de gran importancia para la sostenibilidad de los ecosistemas terrestres y nuestra propia supervivencia. Por desgracia, la contaminación de suelos con metales pesados ha generado un problema medioambiental de gran magnitud, con efectos adversos sobre la funcionalidad y sostenibilidad del ecosistema edáfico. La fitorremediación y la fitogestión se presentan como alternativas de remediación que ofrecen beneficios añadidos a la propia eliminación del riesgo ocasionado por los contaminantes. Por otra parte, las propiedades edáficas que reflejan la actividad, biomasa y diversidad de las comunidades microbianas son indicadores idóneos de la salud del ecosistema edáfico.

En este trabajo, se evaluó el impacto de la contaminación por metales pesados, así como la eficacia de técnicas fitorremediadoras (fitorremediación asistida con enmiendas orgánicas y bacterias endófitas con características promotoras del crecimiento vegetal), en suelos contaminados con metales pesados, mediante la utilización de una serie de propiedades microbianas del suelo y su agrupamiento en categorías de nivel superior, tales como atributos de relevancia ecológica y servicios de los ecosistemas.

Nuestros resultados mostraron que (i) la contaminación reiterada con metales pesados tiene un efecto negativo sobre las propiedades microbianas del suelo y (ii) los procesos de fitoremediación logran mejorar dichas propiedades. La especies *Noccaea caerulescens* y *Rumex acetosa* en procesos de fitoextracción, y *Festuca rubra* en procesos de fitoestabilización, confirmaron el gran potencial que las plantas (pseudo)metalófitas nativas de entornos mineros tienen para la fitorremediación de suelos contaminados con metales pesados. Así mismo, la aplicación de enmiendas orgánicas en procesos de quimio/fitoestabilización tuvo un efecto positivo sobre el crecimiento de las plantas y la salud suelo; sin embargo, este último efecto fue transitorio en el tiempo y, finalmente, resultó en una disminución de la diversidad de las comunidades microbianas edáficas. Si bien la inoculación de plantas en procesos de fitorremediación con bacterias endófitas con características promotoras del crecimiento vegetal no tuvo efecto sobre la biomasa de las plantas, sí mejoró su estado fisiológico, lo que se reflejó en un efecto positivo sobre las propiedades microbianas del suelo. Por último, la estimación de categorías de nivel superior, como atributos de relevancia ecológica y servicios de los ecosistemas, puede aportar información complementaria

acerca de la funcionalidad del sistema edáfico, así como facilitar la interpretación de los resultados.

LABURPENA

Lurzorua sistema dinamiko eta konplexua da, eta bere funtzioak garrantzi handikoak dira lurzoru ekosistemen iraunkortasun eta gure biziraupenerako. Zoritxarrez, metal astunekin kutsaturiko lurzoruek izugarrizko ingurumen arazoa eragin dute, lurzoru ekosistemen funtzionaltasun eta iraunkortasuna kaltetuz. Fitoerremediazioa eta fitogestioa, kutsatzaileek eragindako arriskuak gutxitzeaz gain, beste onura batzuk eskeintzen dituzten erremediazio aukerak dira. Bestalde, komunitate mikrobianoen aktibitate, biomasa eta dibertsitatea isladatzen dituzten lurzoruko propietateak lurzoru ekosistemaren osasunaren adierazle egokiak dira.

Lan honetan, metal astunek sorturiko kutsaduraren eragina eta fitoerremediazio tekniken (medeapen organikoz eta landareen hazkuntzarako ezaugarri sustatzaileak dituzten bakterio endofitoz lagundutako fitoerremediazioa) eraginkortasuna aztertu ziren, metal astunez kutsaturiko lurzoruetan, lurzoruko propietate mikrobianoen erabileraren bidez, eta baita hauek maila goragoko kategoriatan taldekatuz, garrantzi ekologikodun ezaugarriak eta zerbitzu ekosistemikoak.

Gure emaitzek erakutsi dute (i) metal astunen kutsadura errepikakorrak eragin negatiboa dutela lurzoruko propietaten mikrobianoengan eta (ii) prozesu fitoerremediatzaileek propietate hauek hobetzen dituztela. Meatze inguruetako jatorrizkoak diren landare (pseudo)metalofitoek metal astunekin kutsaturiko lurzoruen fitoerremediaziorako duten potentzial handia baieztatu zuten (*Noccaea caerulescens* eta *Rumex acetosa* espezieek fitoerauzketa prozesuetan, eta *Festuca rubra*-k fitoegonkortze prozesuetan). Aldi berean, kimio/fitoegonkortze prozesuetan medeapen organikoen erabilerak eragin onuragarria izan zuen landareen hazkuntzan eta lurzoruaren osasunean; hala ere, azken ondorio hau aldi baterako izan zen eta, azkenerako, lurzoruko komunitate mikrobianoen dibertsitatearen galera eragin zuen. Fitoerremediazio prozesuetako landareak hauen hazkuntzarako ezaugarri sustatzaileak dituzten bakterio endofitoz inokulatzeak beraien biomasan eraginik izan ez zuen arren, bai hobetu zuten landareen egoera fisiologikoa, eta honela lurzoruko propietate mikrobianoetan efektu positiboa izan zuen. Azkenik, maila goragoko kategorien estimazioak, garrantzi ekologikodun ezaugarri eta zerbitzu ekosistemikoak kasu,

informazio osagarria eman lezake lurzoru sistemaren funtzionaltasunari buruz, emaitzen interpretazioa ere erraztuz.

ABSTRACT

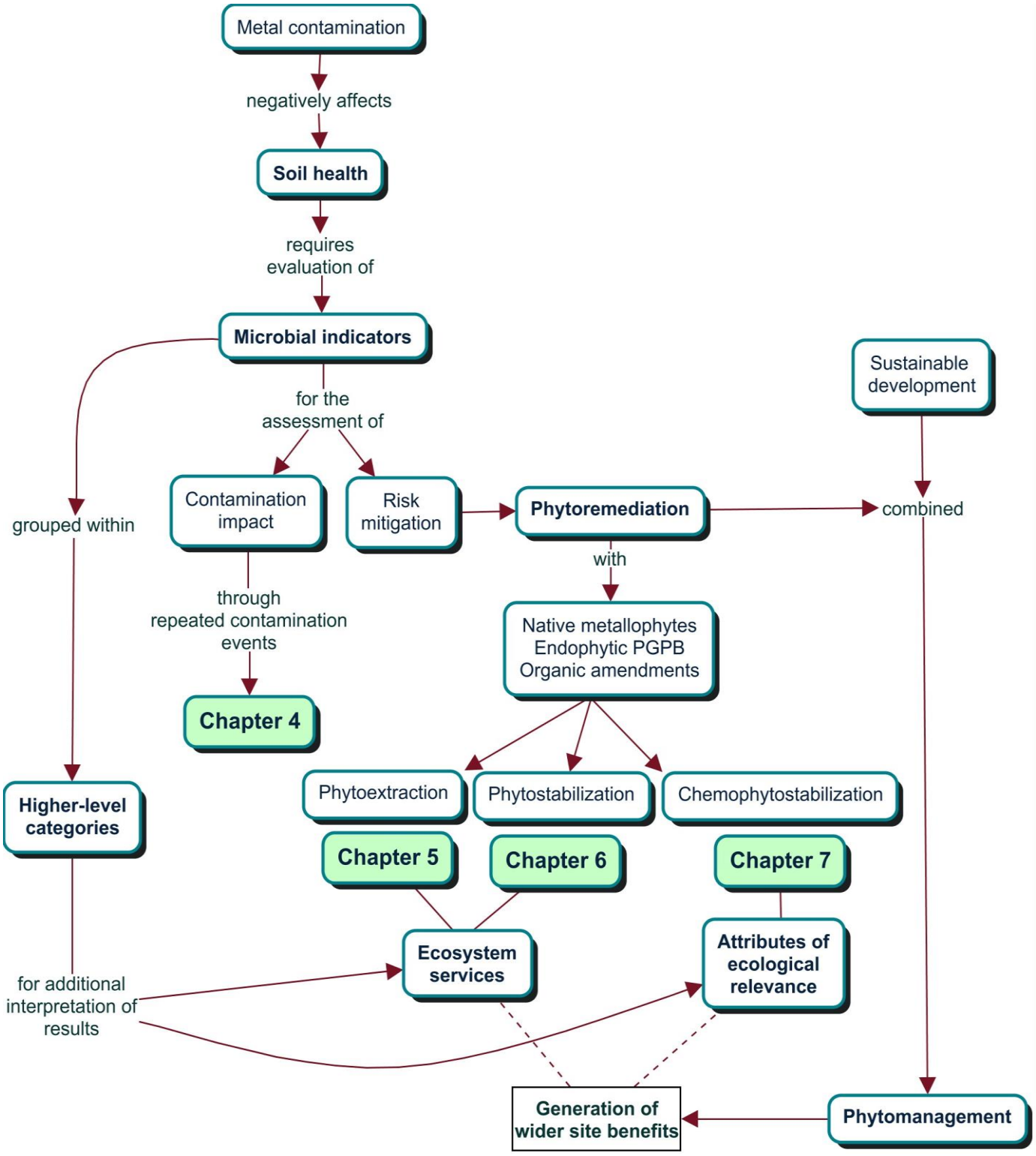
Soil is a dynamic and complex living system whose functions are essential for the sustainability of terrestrial ecosystems and our own survival. Regrettably, the contamination of soils with heavy metals has generated an environmental problem of great magnitude, with deleterious effects on the functioning and sustainability of the soil ecosystem. Phytoremediation and phytomanagement are suggested as remediation options that, in addition to the reduction of the risk generated by the contaminants, offer additional benefits. On the other hand, soil properties that reflect the activity, biomass and diversity of microbial communities are suitable indicators of soil health.

In this work, we evaluated the impact of heavy metal contamination, as well as the effectiveness of phytoremediation techniques (assisted phytoremediation using organic amendments and bacterial endophytes with plant growth-promoting traits) in soils contaminated with heavy metals, through the utilization of a set of soil microbial properties and their grouping within higher-level categories, such as attributes of ecological relevance and ecosystem services.

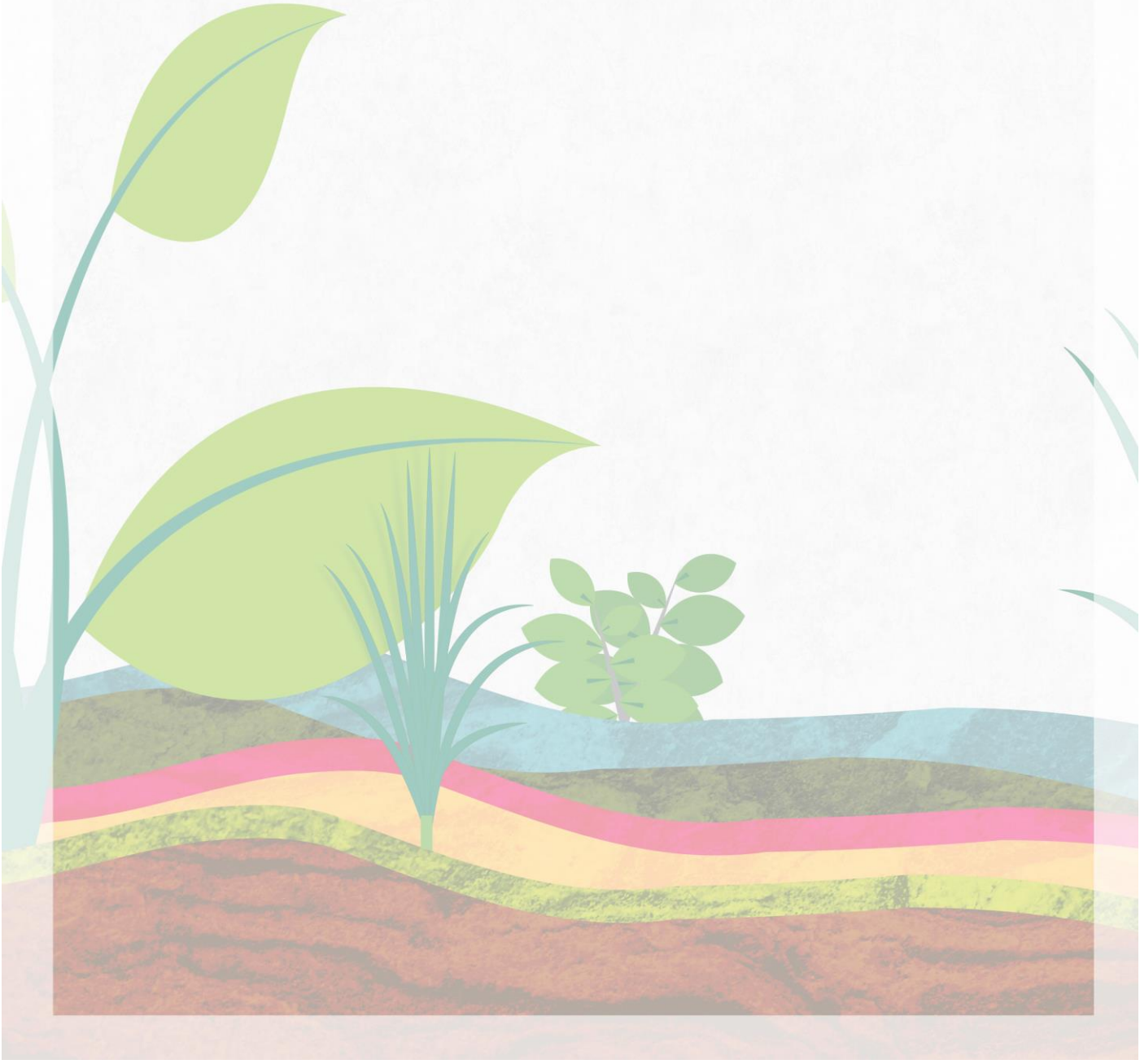
Our results showed that (i) repeated contamination with heavy metals has a negative effect on soil microbial properties, and (ii) phytoremediation manages to improve these properties. The use of *Noccaea caerulescens* and *Rumex acetosa*, in phytoextraction processes, and *Festuca rubra*, in phytostabilization processes, confirmed the great potential of (pseudo)metallophytes, native from mining environments, for the phytoremediation of soils contaminated with heavy metals. Also, the application of organic amendments in chemophytostabilization processes had a positive effect on plant growth and soil health; however, this latter effect was transient over time and, ultimately, it resulted in a reduction of the diversity of soil microbial communities. Even though the inoculation of plants with endophytes with plant growth-promoting traits, in phytoremediation processes, had no effect on plant biomass, it did improve their physiological status, which was reflected in a positive effect on soil microbial properties. Finally, the estimation of higher-level categories, such as attributes of ecological relevance and ecosystem services, can provide complementary

information regarding the functioning of the soil system, and also facilitate the interpretation of results.

CONCEPT MAP


Metal contamination
negatively affects
Soil health
requires evaluation of
Microbial indicators
for the assessment of
Contamination impact
Risk mitigation
Phytoremediation
Sustainable development
combined
grouped within
through repeated contamination events
Chapter 4
with
Native metallophytes
Endophytic PGPB
Organic amendments
Higher-level categories
Phytoextraction
Phytostabilization
Chemophytostabilization
Chapter 5
Chapter 6
Chapter 7
Ecosystem services
Attributes of ecological relevance
for additional interpretation of results
Generation of wider site benefits
Phytomanagement

01 | INTRODUCCIÓN



1. INTRODUCCIÓN GENERAL

Burges A, Alkorta I, Epelde L, Garbisu C. ***From phytoremediation of soil contaminants to phytomanagement of ecosystem services in metal contaminated sites.*** *Submitted.*

1.1 Introduction

Soil health is critical for the appropriate functioning of terrestrial ecosystems (Doran and Parkin, 1996; Doran and Zeiss, 2000). Regrettably, at the moment, different sources of environmental stress are leading to soil degradation processes, such as salinization, erosion, sealing, contamination, loss of organic matter and biodiversity, etc. (Epelde et al., 2009b). In particular, soil metal contamination is a worldwide problem of great magnitude, owing to their toxicity, persistence, bioaccumulation and biomagnification throughout the food chain (Gómez-Sagasti et al., 2012). Metal contamination is adversely affecting soil health at a global scale, with deleterious impacts on ecosystem services.

Physicochemical methods for the remediation of contaminated soils are usually expensive and often result in a deterioration of the soil ecosystem. Therefore, in the last years, the development of environmentally-friendly biological technologies to economically remediate these soils has been stimulated (Hernández-Allica et al., 2006; Gómez-Sagasti et al., 2012). Although physicochemical methods, particularly excavation followed by disposal in controlled landfills ("dig and dump"), are still the most commonly used techniques for the remediation of contaminated soils, in some European countries like The Netherlands, Lithuania and Belgium, *in situ* biological treatments account for up to 20, 13 and 12% of the applied remediation measures, respectively (van Liedekerke et al., 2014). Among these *in situ* biological techniques, bioremediation, a non-destructive, cost-efficient remediation technology, uses the capacity of microorganisms to degrade contaminants. But microorganisms present a critical limitation for the remediation of metal contaminated sites: metals cannot be biodegraded. Microorganisms can transform metals from one oxidation state or organic complex to another, but not remove them from the contaminated soil (Garbisu and Alkorta, 2001).

Interestingly, plants frequently present higher metal tolerance than many microorganisms used for bioremediation (Seth, 2012) and, most importantly, they can (i) literally extract metals from contaminated soils and/or (ii) immobilize them through

sorption by roots, precipitation, complexation or metal reduction in the rhizosphere (Raskin et al., 1997; Ali et al., 2013). Phytoremediation (*i.e.*, the use of green plants to remediate contaminated environments) is a most promising phytotechnology for the remediation of metal contaminated soils. Metallophytes, plants that have evolved mechanisms to tolerate high concentrations of metals, are considered an optimal choice for phytoremediation (Whiting et al., 2001; Boularbah et al., 2006). Based on their physiological strategies to cope with metal contamination, metallophytes can be classified as follows: (i) *accumulators*: they display an active metal uptake and translocation to aerial parts; (ii) *indicators*: they regulate metal uptake so that internal concentrations reflect external soil concentrations; and (iii) *excluders*: they restrict the entry of metals into the root and/or their transport to the shoots (Baker, 1981; Barrutia et al., 2011). Some metallophytes, termed *hyperaccumulators*, have specialized mechanisms that enable them to accumulate metals over 1% of their dry weight, in some cases reaching up to 10% (Baker et al., 2000).

The selection of plant species for phytoremediation depends, among other factors, on the specific type of contaminant, the characteristics of the contaminated site, and the choice of phytoremediation strategy. Interestingly, the revegetation of contaminated sites during phytoremediation helps improve the physicochemical and biological properties of the contaminated soil, by increasing its organic matter content, nutrient levels, biological activity, etc. (Arienzo et al., 2004).

The two main strategies for metal phytoremediation are: (i) *phytoextraction*, the use of plants to extract metals from soil; and (ii) *phytostabilization*, the use of tolerant plants to reduce metal bioavailability (Garbisu and Alkorta, 2001; Ernst, 2005). *A priori*, it seems that nothing much has changed in the last two decades, as these are the two main strategies already mentioned 20 years ago in some of the classical papers on phytoremediation: for example, Salt et al. (1995) emphasized that two subsets of this phytotechnology were applicable to soil metal remediation: (1) phytoextraction, or the use of metal accumulating plants to remove metals from soil; (2) phytostabilization, or the use of plants to reduce metal bioavailability in soil. These two strategies were also described in detail in the excellent review by Salt et al. (1998). Similarly, Cunningham et al. (1995) stated that two approaches to plant-based remediation were being pursued: *decontamination*, where plants and their associated microflora are used to eliminate the contaminant from the soil; and *pollutant-stabilization and containment*, where soil conditions and vegetative cover are manipulated to reduce the environmental hazard.

But nothing could be further from the truth as the field of phytoremediation has much evolved since then, as we shall revise here. Figure 1.1 shows how research on phytoremediation has increased in the last years. This interest in phytoremediation has resulted in progressive research and development of new phytotechnologies, as well as an extensive debate and dissemination of new approaches and theories regarding their use.

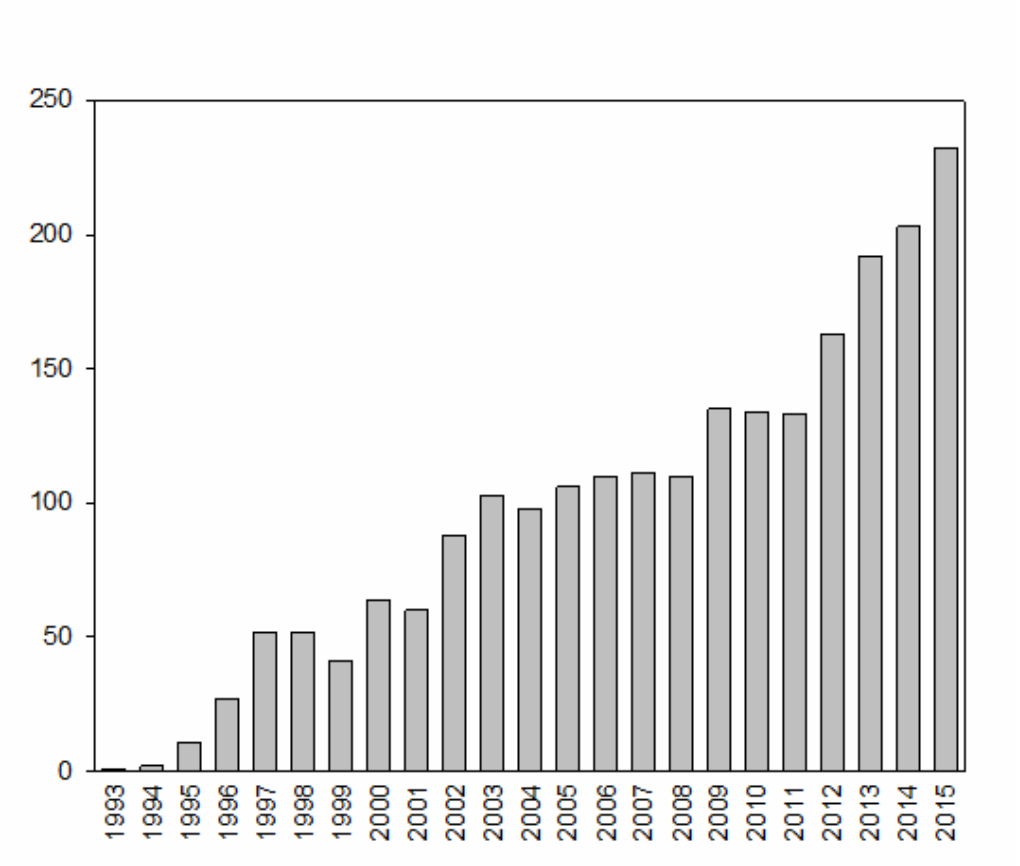


Figure 1.1: Number of articles on phytoremediation per year, based on the number of entries obtained in Web of Science with the term "phytoremediation" on the title.

1.2 Phytoextraction

The inspiration for phytoextraction originated from the discovery of plants, often endemic to naturally mineralized soils, with the capacity to accumulate high amounts of metals in their tissues. For a long time, this capacity was considered a detrimental trait, since these plants represent a risk of introducing toxic metals into the food chain. But later, these plants were identified as suitable candidates for *continuous phytoextraction* (Cunningham et al., 1995; Salt et al., 1995), a strategy based on the use of plants that

naturally accumulate large concentrations of metals in their foliage, with hyperaccumulators being highlighted as the most promising candidates.

1.2.1 Phytoextraction with hyperaccumulators

With more than 400 plant species identified as hyperaccumulators (Boularbah et al., 2006), in the last decades, a great number of phytoextraction studies has been performed using hyperaccumulators. *Noccaea caerulescens* (f.k.a. *Thlaspi caerulescens*) is probably the most extensively studied hyperaccumulator (Baker et al., 1994; Brown et al., 1994; Brown et al., 1995; Robinson et al., 1998; Schwartz et al., 2003; Hernández-Allica et al., 2006; Epelde et al., 2010a). *Noccaea caerulescens* has a remarkable capacity to accumulate zinc (Zn) and cadmium (Cd) in its aboveground tissues. Another member of the *Brassicaceae* family, *Arabidopsis halleri*, is known for its Zn hyperaccumulating capacity (Bert et al., 2000; Zhao et al., 2000) and has also been reported to accumulate Cd at high concentrations (Kupper et al., 2000). On the other hand, the discovery of the arsenic (As) hyperaccumulator fern *Pteris vitata* by Ma et al. (2001) led to intensive screening of the *Pteris* family, resulting in the discovering of other As hyperaccumulator species in this family. Among the great number of species identified as nickel (Ni) hyperaccumulators, many *Alyssum* species have been studied for their Ni phytoextraction potential (Chaney et al., 1997; Robinson et al., 1997; Li et al., 2003).

Hyperaccumulators are unique in terms of their capacity for metal uptake and translocation to the shoots. Hyperaccumulation was probably evolved, from metal tolerance, as an adaptive trait offering a new niche to plants (Assunção et al., 2003). Such is the adaptation of hyperaccumulators to metalliferous environments that Whiting et al. (2000) provided evidence suggesting that *N. caerulescens* exhibited preferential root proliferation in the Zn and Cd rich patches in soil, indicating root metal foraging.

A variety of physiological mechanisms underlie the unique characteristics of hyperaccumulators:

(i) *Metal mobilization*, which is believed to occur through rhizosphere acidification or exudation of mobilizing compounds (Raskin et al., 1997). Microorganisms in the rhizosphere of hyperaccumulators, including root-colonizing bacteria and mycorrhiza, appear to have a role in metal mobilization and availability. Whiting et al. (2001) found that rhizosphere acidification by microorganisms enhanced Zn bioavailability and accumulation in *N. caerulescens*. On the other hand, the secretion

of chelates into the rhizosphere, frequently mediated by microorganisms, and subsequent uptake of the metal-chelate complexes has been described to facilitate metal mobilization by plants (Clemens et al., 2002).

(ii) *Detoxification*, through chelation inside the plant and vacuolar compartmentalization (Wu et al., 2010). Chelation with certain ligands (*e.g*., malate, histidine, citrate, nicotinamine) routes metals to the xylem, promoting root-to-shoot translocation; phytochelatins and metallothioneins route metals towards intracellular sequestration in organelles (Clemens et al., 2002; Eapen and D'Souza, 2005).

(iii) *Transporter proteins*, associated with the uptake, intracellular sequestration and redistribution of metals in plants, *e.g*. the CPx-type metal ATPases, the natural resistance-associated macrophage (Nramp) proteins, the cation diffusion facilitator (CDF) family proteins, and the zinc-iron permease (ZIP) transporter proteins (Pence et al., 2000; Williams et al., 2000; Assunção et al., 2001; Lombi et al., 2002b). Genes encoding for ZIP transporters have been identified in *Arabidopsis thaliana* (Grotz et al., 1998). In *N. caerulescens*, genes of the ZIP family were identified and cloned (ZNT1, ZNT2 and ZTP1); interestingly, these genes are highly expressed in *N. caerulescens* root tissues, whereas in the non-accumulator *Thlaspi arvense* these genes are expressed only under Zn deficiency (Pence et al., 2000; Assunção et al., 2001), suggesting that an alteration in the Zn-induced transcriptional down-regulation of Zn transporter genes can play a role in Zn uptake and hyperaccumulation in *N. caerulescens* plants.

1.2.2 Phytoextraction with high biomass crops

Hyperaccumulators commonly have a low biomass production and growth rate. Accordingly, although high biomass crops accumulate significantly lower concentrations of metals in their aboveground tissues, their use might result in an overall metal removal comparable or even higher to that of hyperaccumulators (Ebbs et al., 1997; Kayser et al., 2000). Emphasis has been placed on crops of the *Brassicaceae* family related to wild metal (hyper)accumulators. Thus, *Brassica juncea* and *B. napus* have been extensively studied for their capacity to accumulate metals (Kumar et al., 1995; Ebbs et al., 1997).

Trees have also been suggested for phytoextraction. Some varieties of *Salix* spp. show a greater (5-fold higher) capacity for Cd phytoextraction than *N. caerulescens* and *Alyssum murale*, due to high biomass production and enhanced transport to the shoots (Greger and Landberg, 1999). Other tree species, *e.g. Betula*, *Populus*, *Alnus* and *Acer*,

have been studied for phytoextraction, owing to their high metal tolerance and accumulation capacity (Pulford and Watson, 2003).

1.2.3 Phytoextraction: challenges and developments

Phytoextraction is certainly an aesthetically-pleasing, environmentally-friendly phytotechnology that has important advantages, such as its low cost, high public acceptance and the possibility of metal recycling from the ashes after phytomass incineration (Vangronsveld et al., 2009; Seth et al., 2011). Unfortunately, a crucial limitation of phytoextraction (according to many, its Achiles´ heel) is the very long time required to effectively extract metals from contaminated soils, particularly in medium and highly contaminated sites (Zhao et al., 2003). But, if the aim is to strip the bioavailable metal fraction from soil ("bioavailable contaminant stripping"), not to reach total metal concentration targets established by legal frameworks, the time for successfully completing the task will be much shorter (Vangronsveld et al., 2009). The low biomass and slow growth of most hyperaccumulators are, to a great extent, responsible for the abovementioned long time required. Fertilization can increase biomass yield, thus enabling plants to access and deplete larger pools of bioavailable metals as a consequence of root proliferation (Robinson et al., 1997; Schwartz et al., 2003; Barrutia et al., 2009). However, in many cases, the moderate increment in the amount of metal phytoextracted, added to the costs and logistic difficulties associated with its large-scale application, hampers the deployment of fertilization in the field.

Other limitations of plants used for phytoextraction are: (i) root depth (which frequently limits applicability to surface soils), (ii) lack of well-known agronomic practices, and (iii) their capacity to accumulate only one metal, when many contaminated soils contain several metals (Garbisu and Alkorta, 2001). Notwithstanding, a low metal bioavailability is another major limiting factor for phytoextraction (Ali et al., 2013). As already mentioned, hyperaccumulators present different mechanisms to solubilise and mobilize metals in order to facilitate their uptake; however, metal bioavailability in soil might still be very limited due to low solubility in water, adsorption onto soil particles and organic matter, etc. (Marques et al., 2009).

It is a well-known fact that the application of chelating agents to soil can increase metal uptake and root-to-shoot translocation (a prime requirement for phytoextraction), thus opening the possibility of using high biomass non-accumulators

for phytoextraction. This strategy is termed *chelate-induced phytoextraction.* Chelating agents prevent precipitation and sorption of metals in soil, thereby maintaining their phytoavailability (Salt et al., 1995). In an attempt to overcome the abovementioned Achiles´ heel, many studies on chelate-induced phytoextraction have been conducted using high biomass plants, especially with ethylenediaminetetraacetic acid (EDTA) and lead (Pb), one of the least phytoavailable metals (Blaylock et al., 1997; Huang et al., 1997; Puschenreiter et al., 2001). The use of chelates for phytoextraction of other metals, such as Cd, Cu and Zn, has also proven successful (Luo et al., 2005). Studies on chelate-induced phytoextraction have also been conducted using moderate biomass accumulators, such as *Rumex acetosa* (Barrutia et al., 2010), or hyperaccumulators such as *Thlaspi goesingense* (Puschenreiter et al., 2001). But an important limitation of chelate-induced phytoextraction is the possibility of promoting metal leaching to other environmental compartments (*e.g.*, groundwater). Wenzel et al. (2003) observed that leaching metal concentrations far exceeded EDTA-induced metal uptake by plants. Furthermore, some chelating agents are phytotoxic at high concentrations. Similarly, the presence of chelates can reduce the abundance and diversity of rhizosphere microorganisms (Marques et al., 2009; Ali et al., 2013). A lot of research has been carried out on the search for less toxic, more biodegradable chelates [*e.g.*, ethylenediamine-N,N'-disuccinic acid (EDDS), nitrilotriacetic acid (NTA), citric acid (CA)], as well as on soil acidification to increase metal bioavailability (Kayser et al., 2000; Puschenreiter et al., 2001; Alkorta et al., 2004). In any event, field application of chelate-induced phytoextraction is still hampered by the abovementioned increased risk of metal leaching.

In parallel, a lot of emphasis has been placed on exploring the possibility of introducing hyperaccumulation traits to fast growth, high biomass plants in an attempt to make phytoextraction a commercial practice (Baker et al., 1994; Brown et al., 1995). After all, an ideal plant for phytoextraction should have a high biomass, fast growth, metal tolerance, and high metal accumulation capacity in its aboveground tissues. Therefore, much interest has been generated on the molecular basis of metal tolerance and accumulation in hyperaccumulators, with the aim of engineering fast-growing, high-biomass plants with the unique properties of hyperaccumulators. Not surprisingly, one approach has been to develop transgenic plants with the desired traits. Some plants, such as, for instance, *B. juncea* and *A. thaliana*, have been genetically modified to increase their biomass and/or metal accumulation capacity (Gasic and Korban, 2007;

Khoudi et al., 2013). Research conducted on the transfer or overexpression of metal transporter genes (van der Zaal et al., 1999; Song et al., 2003), as well as genes involved in the synthesis of metallothioneins, phytochelatins and other chelators (Evans et al., 1992; Zhu et al., 1999a; 1999b), led to enhanced plant metal tolerance and uptake. By overexpression of natural chelators, both plant metal uptake and translocation through the xylem can be facilitated (Wu et al., 2010). Particularly, numerous attempts have been made to boost phytochelatin formation by overexpressing enzymes involved in the synthesis of gluthatione (the phytochelatin precursor), which might also play a role in the plant´s defensive response against metal-induced oxidative stress (Seth et al., 2011). The use of transgenic plants for phytoextraction has shown promising results, but the observed improvements are not good enough to promote their commercial application. Inevitably, the debate on the use of genetically modified plants has affected research on this topic. Indeed, taking in consideration the legal and societal restrictions regarding the use of transgenic plants, conventional breeding and similar traditional methodologies have been seen by many as a promising alternative to genetic transformation: Nehnevajova et al. (2007) used chemical mutagenesis to improve yield and metal uptake of *Helianthus annuus*; similarly, somatic hybridization between *N. caerulescens* and *B. napus* successfully produced hybrids that tolerated high concentrations of Zn (Brewer et al., 1999). Nonetheless, the application of these methodologies for phytoextraction beyond pilot-scale has not been successful.

A most promising alternative to overcome the Achiles´ heel of phytoextraction (*i.e.*, the long time required) is to use plant species that provide an added value in order to obtain economic benefit during the phytoextraction process itself. Energy crops, such as *Miscanthus* spp., *Ricinus communis*, *Jatropha curcas*, *B. napus* and *Cynara cardunculus*, among many others, have been proposed due to their metal tolerance and accumulation capacity along with their usefulness for biofuel production (Llugany et al., 2012; Pandey et al., 2012; Ruiz-Olivares et al., 2013; Pidlisnyuk et al., 2014; Pandey et al., 2016), thus addressing both contaminant remediation and renewable energy demand simultaneously. Other commercial applications of plants used in phytoremediation, such as biochar production, raw materials for industries (paper, bio-chemicals, essential oils, etc.) and medicinal purposes are being studied (Pandey et al., 2016). The use of fast growing trees offers the possibility to combine, for instance, metal extraction with production of biomass for bioenergy and other end-products (*e.g.*, timber, resin, adhesives, etc.) (Schroder et al., 2008). An interesting strategy to facilitate the

commercial application of phytoextraction, termed *phytomining*, rests on the possibility of recovering high value metals from the metal-laden plants used for phytoextraction. It has been claimed (Jiang et al., 2015) that metal recovery from plant biomass increases considerably the economic viability of phytoextraction, while simultaneously eliminating the need for disposal of the contaminated biomass. Chaney et al. (2007) demonstrated that phytomining of Ni can be highly profitable in Ni-contaminated soils. However, a lot of debate still continues on the viability and fate of phytomining, especially regarding its economic viability.

Nowadays, within the phytoremediation field, much attention is being paid to the utilization of plant-associated microorganisms (rhizosphere microorganisms and endophytes) to increase plant growth and metal tolerance and accumulation. It has long been known that arbuscular mycorrhizal fungi (AMF) increase the absorptive surface area of plant roots, thanks to a hyphal network that explores rhizospheres beyond the root-hair zone, thus enhancing water and mineral uptake (Gohre and Paszkowski, 2006). In addition, AMF can promote plant growth through the production of hormones (*e.g.*, cytokinins and gibberellins) and stimulate phytoextraction by increasing metal phytoavailability (Vamerali et al., 2009). At the moment, a lot of research effort is aimed at screening for metal tolerant AMF that could then be used for inoculation of hyperaccumulators to improve phytoextraction efficiency.

Similarly, it has been widely reported that the application of plant growth-promoting rhizobacteria (PGPR) and endophytes (bacteria that colonize the internal tissues of plants) has large potential for phytoremediation, owing to their ability to increase plant growth and contaminant tolerance and uptake (Mastretta et al., 2009; Chen et al., 2010; Luo et al., 2011; Ma et al., 2011). Plant growth-promoting bacteria produce compounds (*e.g.*, phytohormones, siderophores, antibiotics, enzymes, organic acids, biosurfactants) that stimulate root growth and development, protect plants against pathogens, improve plant nutrient uptake, increase plant tolerance to contaminants and other abiotic stresses, and facilitate contaminant uptake and translocation to aerial parts (Ma et al., 2011). In this respect, 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity has been reported to be a major mechanism used by plant growth-promoting bacteria, through the lowering of stress ethylene (Glick, 2014). In the last years, special attention is being paid to endophytes isolated from metal tolerant plants, which have the potential to elicit physiological changes that modulate the growth and development of plants in metal contaminated sites (Burges et al., 2016). Unfortunately, the application

of plant growth-promoting bacteria under field conditions is frequently discouraging, among other reasons, due to the poor survival and ecological performance of the inoculated bacteria in the contaminated site. Much research is needed on the selection of traits related to the competitive fitness of these bacterial strains before they are applied to contaminated soil. New methodological tools, such as next-generation sequencing (NGS) techniques, will probably shed light on the mechanisms responsible for the ecological fitness of plant growth-promoting bacteria in contaminated soils. Certainly, NGS techniques and new bioinformatic tools are greatly expanding our understanding of plant-microbe interactions during phytoremediation (Bell et al., 2014).

1.3 Phytostabilization

When soils are contaminated with high concentrations of metals and/or a mixture of different metals, phytoextraction is not a feasible option for all the limitations described above (in particular, the long time required). On the other hand, many metal contaminated soils lack an established vegetation cover due to the toxic effects of metals and/or recent physical disturbances, resulting in barren soils that are prone to erosion and metal leaching (Salt et al., 1998). In these cases, phytostabilization can be a cost-effective, environmentally-friendly remediation method. Initially, research on phytostabilization was focused on the search for metal tolerant plants that could then be used for the remediation of highly contaminated mine wastes and bare industrial sites (Smith and Bradshaw, 1979). Plants for phytostabilization should be metal tolerant, have an extensive root system, produce a large amount of biomass, and keep root-to-shoot translocation as small as possible to prevent the entry of contaminants into the food chain (Alkorta et al., 2010; Gómez-Sagasti et al., 2012). Many excluder plants (*e.g.*, *Festuca rubra*, *Agrostis capillaris*, *A. stolonifera*, *Lolium perenne*, *Trifolium repens*) meet these characteristics and have been used for the revegetation of highly contaminated soils (Vangronsveld et al., 1996; Arienzo et al., 2004; Pérez-de-Mora et al., 2006; Bidar et al., 2007; Epelde et al., 2009a). Trees have also been studied for their phytostabilization potential due to their massive and deep root systems and the addition of litter to the surface, resulting in an organic cover that enhances nutrient cycling, soil aggregation and water holding capacity (Pulford and Watson, 2003). Interestingly, the high transpiration rate and water demand of some tree species, like *Salix* spp., helps reduce the downward flow of water through soil, thus lowering the risk of metal leaching (Pulford and Watson, 2003).

An extensive plant cover (i) prevents the dispersion of metal contaminated soil particles by wind and/or water erosion and (ii) decreases metal availability and mobility through root accumulation and rhizosphere-induced adsorption and precipitation (Vangronsveld et al., 2009).

1.3.1 Aided phytostabilization

Highly contaminated sites such as, for instance, mine tailings, are characterized by poor physical structure, low nutrient concentrations and high levels of bioavailable metals, thus complicating the establishment of a vegetative cover (Burges et al., 2016). The application of organic and/or inorganic amendments to reduce metal bioavailability (*i.e.*, chemical stabilization) is frequently used, in combination with metal tolerant plants, during phytostabilization initiatives, among other reasons, to facilitate and enhance revegetation in poor, contaminated soils by improving their physicochemical and biological properties. The incorporation of organic amendments to metal contaminated soil can facilitate plant colonization by increasing its organic matter content and pH value, adding essential nutrients, improving physical characteristics (*e.g.*, water holding capacity and bulk density) and reducing metal bioavailability (Alvarenga et al., 2009a, 2009b; Epelde et al., 2009a). This technology, which combines the application of amendments and revegetation with metal tolerant plants, has been termed "aided phytostabilization" or "chemophytostabilization" (Knox et al., 2000; Alvarenga et al., 2009a; 2009b), and appears most promising for the remediation of sites highly contaminated with metals (Alkorta et al., 2010).

Many amendments have been used in aided phytostabilization, with particular emphasis on the reuse of organic and inorganic wastes to reduce their disposal through revalorization into co-products. Thus, compost from domestic and garden wastes, sewage sludge, farmyard manure and animal slurry, municipal solid waste, litter, leonardite, etc. have been used for aided phytostabilization (Pérez-de-Mora et al., 2006; Ruttens et al., 2006; Alvarenga et al., 2009a; 2009b; Epelde et al., 2009a). Commonly used inorganic amendments are phosphorous compounds, aluminosilicates, metal oxyhydrates, natural and synthetic zeolites, and cyclonic and fly ashes (Lombi et al., 2002a; Kumpiene et al., 2009; Alkorta et al., 2010). Lime ($CaCO_3$) application aims at increasing soil pH to decrease the bioavailable metal fraction (Pérez-de-Mora et al., 2006; Epelde et al., 2014b).

The use of amendments has to be carried out with caution as amendments can have undesirable effects: for instance, an inappropriate use of organic amendments can result in underground water contamination by nitrates, antibiotics, hormones and loss of soil biodiversity, posing a risk to environmental and human health (Goss et al., 2013; Burges et al., 2016). Organic and inorganic amendments can induce other negative effects like destruction of soil structure, addition of toxic compounds, immobilization of essential nutrients, etc. (Alkorta et al., 2010). Moreover, although amendments have demonstrated to aid revegetation, plant roots may not extend readily from a fertile layer into underlying non-amended contaminated soil (Pulford and Watson, 2003), limiting the potential of this phytotechnology to the top layer of soil.

Interestingly, microorganisms living in the rhizosphere of plants may contribute to stabilize metals, thus assisting in the revegetation process. Bacteria and mycorrhiza have been reported to have a role in the immobilization of metals in soil, by actively changing metal speciation, adsorbing metals onto their cell walls or through precipitation processes (Gohre and Paszkowski, 2006; Ma et al., 2011). AMF can serve as a filtration barrier against metal transfer to the aerial parts (Gohre and Paszkowski, 2006); in turn, bacterial endophytes can produce chelators, such as siderophores, and then affect the translocation of metals to shoots, inducing their accumulation in the roots (Mastretta et al., 2009). As described above for phytoextraction, lots of research is being carried out on the use of PGPR and endophytes not only to enhance plant growth and metal tolerance, but also to increase root surface and depth for phytostabilization initiatives (de-Bashan et al., 2010; Ma et al., 2011; Glick, 2014; Burges et al., 2016).

In any case, it is always important to take into consideration the logistics and costs associated with the management of amendments, bioinoculants (AMF, PGPR, endophytes) and agronomic practices that may be required for the implementation of (aided) phytostabilization in certain contaminated soils, due to, for instance, the extensiveness and remoteness of many of these sites.

The choice of the appropriate plants, taking into account the environmental and physicochemical characteristics of a specific site, is a crucial aspect of phytostabilization. Revegetation with native plant species adapted to the harsh conditions prevailing in these sites, can be effective for phytostabilization without the aid of amendments (Bidar et al., 2007; Burges et al., 2016). But, in some cases, the application of amendments is unavoidable, particularly in sites with a lack of a seed bank of nearby native plants or a disperse vegetation cover (de-Bashan et al., 2010).

1.3.2 Bioavailable versus total metal concentrations

One of the most important obstacles for phytostabilization is that environmental agencies are most frequently focused on total metal concentrations and tend not to be concerned about metal bioavailability. But estimations of total metal concentrations appear poorly indicative of the recovery of soil quality during metal phytoremediation, as soil quality improvements do not necessarily result from a decrease in total metal concentration. It is generally accepted that the adverse impact of metals on soil functioning is related to mobile/bioavailable elemental pools rather than total metal concentrations (Kumpiene et al., 2009). On the other hand, many times, bioavailable concentrations have no correlation with total concentrations (Burges et al., 2015). Metal bioavailability is more important for environmental protection and risk assessment than total metal concentrations because it represents the labile fraction subject to leaching and uptake by soil organisms (Madejón et al., 2006).

Regrettably, there is a lack of agreement on the best way to estimate metal bioavailability. Bioavailable metal fractions are generally estimated using chemical extractants, such as neutral salts ($CaCl_2$, $NaNO_3$), mild acids, organic extractants (DTPA and EDTA) and resin-based techniques, as well as several solid and solution phase speciation methods, all with disputed success owing to the variability of metal bioavailability in space and over time (Menzies et al., 2007). Nonetheless, there seems to be some consensus that neutral salt extractants, and particularly $CaCl_2$, are likely to be most suitable for predicting metal phytoavailability (Madejón et al., 2006; Menzies et al., 2007).

But metal stabilization through (aided) phytostabilization is not necessarily permanent owing to the risk of a reversal of metal immobilization over time. Then, (aided) phytostabilization must not be considered as the final solution for a contaminated site; on the contrary, it must be thought as a temporary management or "holding strategy" for stabilizing metal contaminants while planning a more definitive remediation (Vangronsveld et al., 2009; Cundy et al., 2016).

In contaminated sites where, for whatever reason, it is imperative to reach specific total metal concentration targets in a short time, the use of a tiered remedial approach, in which physicochemical methods are first used to reduce metal concentrations quickly and then phytoremediation is applied as polishing step, is recommended (Alkorta et al., 2010). Physicochemical methods are more effective at

removing metals from soil but their "side effects" generally result in a deterioration of the soil ecosystem. By using this tiered approach, once a reduction in soil metal concentrations has been achieved, phytoremediation strategies might counterbalance those negative side effects on soil functioning. Indeed, although phytoremediation is not effective at quickly removing metals from soil, it is an ideal technique to relatively rapidly improve soil quality.

As a means of overcoming some of the abovementioned limitations, the concept of "ecosystem services" is nowadays being used to provide added value to the benefits resulting from revegetation of contaminated sites, in terms of erosion control, carbon sequestration, biomass production, aesthetic value, etc. The idea is that the provision of ecosystem services may compensate the inconveniences raised by those limitations. According to this paradigm, (aided) phytostabilization should be considered as a management strategy for contaminated sites which offers economic, environmental and societal benefits (Cundy et al., 2016) (Figure 1.2). Thus, a shift from phytoremediation strategies to phytomanagement options is required, in which remediation strategies are combined with sustainable site management options, resulting in a net gain (or at least no gross reduction) in soil functions and ecosystem services, as well as achieving effective risk management (Cundy et al., 2016).

1.4 Phytomanagement

Phytomanagement is based on the long-term combination of profitable site uses with gentle remediation options (GROs), leading not only to reduction of contaminant linkages (*e.g.*, soil-food pathways) but also to the restoration and/or generation of wider site services (Cundy et al., 2016) (Figure 1.2). GROs involve many technologies, including the aforementioned remediation methods based on the use of plants, fungi and/or bacteria, with or without the addition of amendments.

While phytoremediation aims at providing risk management, through the removal and/or immobilization of the bioavailable metal pool fraction by, for instance, phytoextraction or phytostabilization methods, phytomanagement approaches encourage the use of GROs as part of an integrated site management, in which, along with risk mitigation, the accomplishment of economic, social and environmental benefits with value for humankind is taken into consideration. Some of the benefits provided by the use of GROs include water resource improvement, provision of green space, soil erosion prevention, surface and groundwater flow management, renewable energy and

material generation, reduction of soil sealed surface area, greenhouse gas mitigation and carbon sequestration, recovery of land values, amenity and leisure, etc. (Kidd et al., 2015; Cundy et al., 2016).

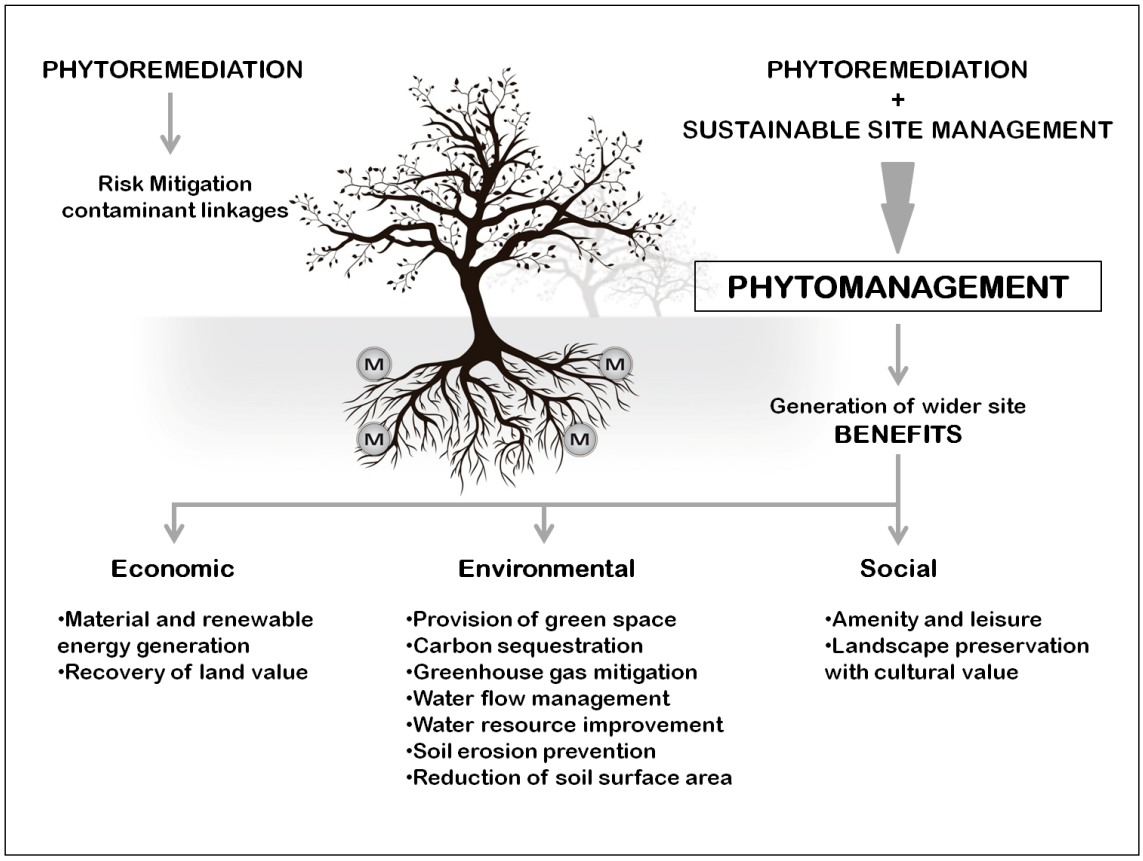


Figure 1.2: Schematic representation of the phytomanagement approach.

But phytomanagement seems to meet the same obstacles confronted by phytoremediation, such as, for example, the legal frameworks that predicate removal of metal contaminants to reach total concentration targets, the need for long-term monitoring, reproducibility uncertainty due to the complexity of the soil ecosystem, etc. Then, many stakeholders have the perception that GROs are slow, logistically not feasible or more suited to large and marginalized areas with low land value (Vangronsveld et al., 2009; Mench et al., 2010; Kidd et al., 2015; Cundy et al., 2016). Therefore, information on successes and failures of phytomanagement options at the field scale must be collected, in order to (i) determine the long-term ecological, ecotoxicological, social and financial sustainability of these phytotechnologies, and (ii) demonstrate their efficiency in order to promote their use and encourage participation of stakeholders (Mench et al., 2010; Gutiérrez et al., 2015; Kidd et al., 2015).

Phytomanagement approaches, and the associated provision of economic, environmental and social benefits, also allow the use of phytotechnologies as a "holding strategy" (Cundy et al., 2016), which alleviates pressures and inconveniences due to time uncertainty, until more favourable conditions enable site regeneration.

1.5 Monitoring

The goal of any metal phytoremediation must be not only to remove the metals from the soil or to render them harmless, but also to restore soil functioning (Epelde et al., 2009a). Therefore, the recovery of soil functions and, concomitantly, ecosystem services should lie at the core of the evaluation of the success of a phytoremediation initiative.

This assessment can include the well-known concept and practice of "Ecological Risk Assessment (ERA)-TRIAD methodology", based on the weight of evidence approach, which takes into consideration a chemical, toxicological and ecological line of evidence (Gutiérrez et al., 2015). In any event, in the last years, a lot of interest has been placed on the recovery of "soil quality" (*i.e.*, the capacity of soil to perform its functions) within the phytoremediation field, whose concept has helped attract attention towards soil conservation (Garbisu et al., 2011). Accordingly, it is essential to have a good set of indicators that provide information on soil functioning in order to properly assess the effectiveness of phytoremediation in terms of the resulting improvement in soil quality. In addition to total and bioavailable metal concentrations, other soil physicochemical parameters (*e.g.*, soil pH, organic matter content, cation exchange capacity, texture, etc.) are used to provide information regarding the behaviour of metals in soil. But physicochemical parameters cannot provide direct information on one key aspect of the soil contamination field: the evaluation of the adverse impact of contaminants on soil resident communities. By contrast, biological parameters from soil macrofauna, mesofauna, microfauna and, particularly, microbial communities have great potential as indicators of soil quality. Thus, microbial parameters related to the activity, biomass (abundance), and diversity of soil microbial communities are, more and more, being used as indicators of soil quality due to their sensitivity, rapid response, ecological relevance, and capacity to provide information that integrates many environmental factors (Epelde et al., 2009a). Nevertheless, microbial parameters are context dependent and often difficult to interpret from a soil quality point of view. In an attempt to overcome this problem, Garbisu et al (2011) proposed to link the concept of

soil quality to that of ecosystem health by means of grouping parameters in higher-level categories such as ecosystem services or ecological attributes. Following this line of thought, we carried out a chemical stabilization field experiment (Epelde et al., 2014b), in which we grouped soil microbial properties within a set of ecosystem attributes of ecological relevance, such as vigour, organization, stability, suppressiveness and redundancy, and confirmed the validity of this approach to facilitate the interpretation of the beneficial impact of remediation on soil quality.

Other authors (Velasquez et al., 2007; Rutgers et al., 2012) have also proposed to evaluate phytoremediation options according to the concept of ecosystem services. Again, in order to confirm the validity of this approach, we performed an endophyte-assisted aided phytostabilization study in which we interpreted our results following this line of thought (Burges et al., 2016). To this purpose, we grouped soil physicochemical and biological indicators within a set of ecosystem services (*i.e.*, nutrient cycling, carbon storage, water flow regulation, water purification, contamination control, pest control, fertility maintenance and biodiversity) and validated the use of this approach for the assessment of phytoremediation efficiency. When a management strategy incorporates the concept of ecosystem services, quantifiable soil features can be more easily linked to land-use expectations in a defendable and transparent way (Rutgers et al., 2012).

In any case, one must bear in mind that metal phytoremediation will either need long time to remove the metals from the soil (phytoextraction) or will not remove them at all (phytostabilization). Therefore, there is always a risk that metals can be partially and gradually released, as a result of changes in soil condition over time. Consequently, long-term monitoring follow-up programmes must always be implemented, so that we ensure that the obtained reduction in metal toxicity and recovery of soil quality remain as such (Epelde et al., 2014a). But long-term monitoring programs are exposed to a variety of challenges over time: for instance, new analytical techniques, methods and equipment might appear in the market; novel scientific theories might be accepted; budget fluctuations might occur; new legislation can be implemented, etc. (Epelde et al., 2014a). Therefore, we recently proposed to apply the concept of adaptive monitoring to long-term phytoremediation monitoring programs (Epelde et al., 2014a) and periodically revised the parameters selected for such programs and their interpretation, by means of expert judgement (Epelde et al., 2014a).

1.6 Final remarks

In the last two decades, extensive work has been conducted on the search for phytotechnologies for the remediation and management of metal contaminated sites (*i.e.*, phytoextraction, phytostabilization). Notwithstanding, there are still serious limitations for these phytotechnologies to become efficient and cost-effective on a field and commercial scale.

Although, in the early days of phytoremediation, there was a perception that these phytotechnologies could return contaminated sites to productive use, the limitations described above have caused a shift of paradigm towards the search for the many values (*e.g.*, economic, environmental and societal benefits) that can be obtained from the large-scale application of phytomanagement. This shift in practice from phytoremediation strategies to phytomanagement options has opened the door for reclaiming previously neglected contaminated sites of low development value.

Nonetheless, long-term monitoring follow-up programmes will always be necessary to ensure that risk mitigation and soil quality and ecosystem health improvement remain as such, preferably incorporating the paradigm of adaptive monitoring, which enables monitoring programs to evolve iteratively as new information emerges. These monitoring programs should ideally focus on the following three aspects: (i) contaminants and associated risks, (ii) soil quality and (iii) soil ecosystem services.

Much further research is needed to better understand the (i) biodiversity and ecology of metallophytes and associated microorganisms (AMF, PGPR, endophytes) and (i) interactions between contaminants-soil-plants-microorganisms. Luckily, at present, NGS techniques and new bioinformatic tools are greatly expanding our understanding of these crucial interactions.

02 | HIPÓTESIS Y OBJETIVOS

2. HIPÓTESIS Y OBJETIVOS

2.1 Hipótesis

Las propiedades microbianas edáficas aportan información valiosa sobre la funcionalidad del suelo, por lo que se presentan como indicadores idóneos de la salud del ecosistema edáfico. Análogamente, el agrupamiento de las citadas propiedades microbianas en categorías superiores, tales como atributos de relevancia ecológica y servicios de los ecosistemas, facilita la interpretación de los datos obtenidos y aporta información complementaria acerca de la salud del ecosistema edáfico. En consecuencia, se plantea la siguiente hipótesis:

Las propiedades microbianas del suelo y su agrupamiento en categorías superiores pueden ser unas herramientas idóneas para evaluar (i) el impacto de la contaminación por metales pesados sobre la salud del suelo y (ii) la eficacia de procesos de fitorremediación y fitogestión.

2.2 Objetivo general

El objetivo general de este trabajo fue evaluar el impacto de la contaminación por metales pesados y la eficacia de técnicas fitorremediadoras, a través del empleo de plantas asistidas con enmiendas orgánicas y bacterias endófitas promotoras del crecimiento vegetal, mediante la determinación de una serie de propiedades microbianas del suelo y su agrupamiento en categorías de nivel superior, tales como atributos de relevancia ecológica y servicios de los ecosistemas.

2.3 Objetivos específicos

- Estudiar la evolución temporal de la toxicidad causada por la contaminación reiterada con uno o varios metales simultáneamente, mediante la monitorización de su biodisponibilidad en el suelo (Capítulo 4).
- Evaluar el impacto de la contaminación reiterada, con uno o varios metales simultáneamente, sobre la salud del suelo, mediante la monitorización de propiedades microbianas con potencial indicador de la citada salud (Capítulo 4).
- Estudiar el potencial de especies (pseudo)metalófitas nativas de emplazamientos mineros para procesos de fitoextracción (*Noccaea caerulescens* y/o *Rumex acetosa*) y fitoestabilización (*Festuca rubra*) de suelos contaminados con metales pesados (Capítulos 5 y 6).

- Evaluar el potencial de bacterias endófitas con características promotoras del crecimiento vegetal, aisladas de especies (pseudo)metalófitas nativas de un emplazamiento minero, para procesos fitorremediadores (Capítulos 5 y 6).
- Evaluar la eficacia de un proceso de fitoextracción, con las especies (hiper)acumuladoras *Noccaea caerulescens* y/o *Rumex acetosa*, asistido con bacterias endófitas con características promotoras del crecimiento vegetal, mediante la utilización de propiedades microbianas con potencial indicador de la salud del suelo (Capítulo 5).
- Evaluar la eficacia de un proceso de fitoestabilización con la especie *Festuca rubra*, asistido con enmienda orgánica y/o bacterias endófitas con características promotoras del crecimiento vegetal, mediante la utilización de propiedades microbianas con potencial indicador de la salud del suelo (Capítulo 6).
- Evaluar la eficacia de un proceso de quimioestabilización en campo con enmienda orgánica, mediante la utilización de propiedades microbianas con potencial indicador de la salud del suelo (Capítulo 7).
- Evaluar la eficiencia de distintos procesos remediadores de suelos contaminados mediante la agrupación de propiedades microbianas del suelo en categorías de nivel superior: servicios de los ecosistemas (Capítulos 5 y 6) y atributos de relevancia ecológica (Capítulo 7).

03 | PROCEDIMIENTOS GENERALES

3. PROCEDIMIENTOS GENERALES

La línea de trabajo del presente estudio pivota en torno a la utilización de parámetros microbianos para evaluar la salud de suelos contaminados por metales pesados, y en particular, de suelos de entornos mineros. Una primera parte de este trabajo se realizó con suelo de una pradera de Derio (Bizkaia) que se contaminó de manera artificial, mientras que la mayor parte de los ensayos se han centrado en suelo minero contaminado por metales pesados. En este apartado, se describen: (i) el lugar y las condiciones de estudio empleadas para llevar a cabo los ensayos, (ii) las técnicas analíticas realizadas que representan la herramienta principal para la consecución del objetivo de este trabajo, y (iii) el posterior tratamiento de datos de los resultados obtenidos.

3.1 Lugar de estudio y condiciones experimentales

3.1.1 Lugar de estudio

El lugar en el que principalmente se ha llevado a cabo este estudio corresponde a una antigua explotación de Pb y Zn, bajo el nombre de "mina Txomin", localizada en el municipio de Lanestosa, Bizkaia (latitud 43º3´; longitud 3º 26´). El clima de la zona es templado y húmedo, sin cambios estacionales extremos, con una precipitación anual de 1.400 mm (pudiendo oscilar entre 1.000 y 2.000 mm en función del año), y una temperatura media anual que varía entre los 11 y 15 ºC.

El emplazamiento de la mina está situado en un área montañosa, con su punto más alto a 440 m sobre el nivel de mar y laderas con 30-50% de pendiente. Si bien no se sabe con exactitud cuándo comenzó la actividad minera, parece haber indicios de que la mina ya estaba operativa a principios del siglo XX. Su explotación, basada principalmente en la extracción de Pb, tanto a cielo abierto como en galerías subterráneas, cesó en la década de los 70. En la actualidad el área correspondiente a la antigua mina presenta hoyos abiertos y galerías subterráneas, antiguas zonas de procesado de mineral con escombreras de roca y escoria, balsas para lavado de roca y decantación de lodos, etc. El tipo de extracción, así como la orografía de la zona, han dado como resultado una elevada heterogeneidad en la contaminación del suelo, siendo el Pb, Zn y Cd los principales metales pesados encontrados en dicho suelo.

Figura 3.1: Vistas de la mina "Txomin". Fuente: NEIKER-Tecnalia.

De este modo, las elevadas concentraciones de metales pesados presentes en ciertas zonas de la mina, así como su remota localización (sin presión urbanística e inmobiliaria), el cese de la explotación durante más de 30 años, y la baja actividad antropogénica existente (tan solo cierta actividad ganadera de baja intensidad), han propiciado el desarrollado de una comunidad vegetal característica y peculiar que confieren a este lugar la condición de banco de germoplasma de gran valor ecológico. La vegetación de la mina está compuesta por asociaciones de *Festuca rubra*, la especie más abundante, con *Noccaea caerulescens*. Generalmente, y dependiendo de las zonas y de las concentraciones de metales pesados existentes, esta asociación suele ir acompañada de *Jasione montana*, *Rumex acetosa* y, menos frecuentemente, *Plantago lanceolata*. En las inmediaciones de la mina, donde la contaminación de metales pesados ha sido mucho menor, las especies dominantes son *Ulex europaeus*, *Pteridium aquilinium* y *Molinia caerulea* (Barrutia y cols., 2011). Todo esto hace que la "mina Txomin" sea un emplazamiento idóneo para llevar a cabo estudios de remediación de suelos contaminados por metales pesados y explorar el gran potencial de la biodiversidad existente en la zona para procesos de descontaminación.

El acopio de suelo de la mina, empleado en los ensayos en microcosmos de los Capítulos 5 y 6, se realizó de la siguiente manera: (i) se seleccionó una zona de la mina con contaminación media (con una concentración de Zn entre 10.000 y 20.000 mg kg^{-1}) y una vegetación homogénea dominada por *F. rubra*; (ii) se excavó la tierra hasta una profundidad de 20 cm; (iii) se transportó al laboratorio donde se eliminaron las raíces y

se pasó por un tamiz de 4 mm de diámetro; y, finalmente, (iv) se homogenizó el suelo con una hormigonera.

En el caso de la utilización de suelo contaminado artificialmente, el muestreo se realizó en una pradera de Derio (Bizkaia) siguiendo el mismo procedimiento y, posteriormente, se contaminó de forma artificial con metales pesados, tal y como está descrito en el Capítulo 4.

3.1.2 Condiciones de cultivo y ensayo

Las especies vegetales empleadas en los distintos ensayos también se obtuvieron de la mina de Lanestosa. *Noccaea caerulescens* y *Rumex acetosa* se recolectaron en forma de semillas. En el caso de *Festuca rubra*, se recogieron plantas que, posteriormente, se reprodujeron por esquejes en una mezcla de perlita-vermiculita (1:3).

Las semillas de *N. caerulescens* y *R. acetosa* se germinaron en una cámara de crecimiento en una mezcla de perlita-vermiculita (1:3) humedecida con agua. Al cabo de 4 semanas, las plántulas se trasplantaron individualmente a una mezcla de perlita-vermiculita (2:3), donde se dejaron crecer un mes más, humedeciéndose con una solución nutritiva con las siguientes características:

Compuesto	**Concentración**
$Ca(NO_3)_2 \cdot 4H_2O$	1 mM
$MgSO_4 \cdot 7H_2O$	0.5 mM
K_2HPO_4	0.5 mM
KCl	0.1 mM
MES-HCl buffer pH 6	2 mM
KOH	1 mM
H_3BO_3	10 µM
$Na_2MoO_4 \cdot 2H_2O$	0.2 µM
$MnSO_4 \cdot 4H_2O$	1.8 µM
$CuSO_4 \cdot 5H_2O$	0.3 µM
$NiSO_4 \cdot 6H_2O$	0.5 µM
Fe-EDDHA	100 µM
$ZnSO_4 \cdot 7H_2O$	1 µM/5 µM*

*Para *R. acetosa* / *N. caerulescens*

Las esquejes de *F. rubra* se trasplantaron individualmente a una mezcla de perlita-vermiculita (1:3), humedecida con agua, y se dejaron crecer 4 semanas. En el ensayo con *F. rubra*, también se empleó una variedad comercial de esta especie, cuyas semillas se obtuvieron de "Semillas Silvestres S.L." y se germinaron bajo las mismas condiciones, dejándolas crecer 4 semanas.

Las condiciones de la cámara de crecimiento, tanto durante la germinación y crecimiento de las plantas, como durante los ensayos a escala microcosmos de los Capítulos 5 y 6, fueron las siguientes: ciclo de luz /oscuridad de 14/10 h, temperatura de 20/16 ºC día/noche, 70% de humedad relativa y una intensidad de PAR (radiación fotosintéticamente activa) de 150 µmol fotón m^{-2} s^{-1}.

Figura 3.2: Tiestos en la cámara de crecimiento con (a) *Festuca rubra*; (b) *Noccaea caerulescens* y *Rumex acetosa*. Fuente: NEIKER-Tecnalia.

El ensayo en microcosmos del Capítulo 4 se realizó en oscuridad, a temperatura ambiente (20 ºC) en una habitación interior en las instalaciones de NEIKER-Tecnalia a temperatura y humedad constantes.

Por otro lado, las bacterias endófitas aisladas (Capítulos 5 y 6) se obtuvieron de plantas recogidas en la mina de las siguientes especies: *Festuca rubra*, *Noccaea caerulescens*, *Rumex acetosa*, *Betula alba* y *Salix atrocinerea*. Para el aislamiento y purificación de las cepas bacterianas se siguió el método de Surette y cols. (2003), el cual está descrito en mayor detalle en el Capítulo 6.

3.2 Parámetros analíticos

Al finalizar el periodo de tiempo de los ensayos, el material vegetal fue separado, limpiado y lavado de restos de suelo, para su procesamiento. Por otro lado, el suelo fue, primeramente, pasado por un tamiz de 4 mm de diámetro (del que se guardaba una muestra para la determinación de la capacidad de retención hídrica) y, posteriormente, por otro tamiz de 2 mm. Una vez tamizado, el suelo se conservaba en fresco a 4 ºC para su análisis, excepto una parte, que se conservaba a -20 ºC para análisis moleculares.

En el caso del ensayo de contaminación repetitiva (Capítulo 4), el muestreo se realizó en varios momentos a lo largo del experimento. Para ello, el suelo de las tarrinas se homogenizaba previamente a la toma de la muestra.

3.2.1 Parámetros de planta

La contaminación de metales pesados en suelos conlleva una serie de efectos adversos sobre los organismos vivos. La fitotoxicidad causada por metales pesados tiene efectos inhibitorios sobre las planta a nivel fisiológico que, finalmente, repercuten en su crecimiento y aspecto. Además de la biomasa y los signos visuales (clorosis, senescencia de hojas, etc.), el estado fisiológico de las plantas puede evaluarse mediante el análisis de una serie de parámetros, tales como la eficiencia fotosintética o la cantidad de pigmentos y compuestos antioxidantes. Esta información es muy útil para determinar qué especies o variedades son más idóneas para procesos fitorremediadores. Así pues, en los experimentos de fitorremediación en microcosmos (Capítulos 5 y 6), se realizaron mediciones de la eficiencia fotosintética (Fv/Fm) con un fluorímetro portátil y, además, se determinó la concentración de pigmentos fotosintéticos (clorofila a y b, carotenoides, violaxantina, anteraxantina, zeaxantina, luteína y neoxantina) y antioxidantes lipofílicos (α-tocoferol) mediante HPLC, siguiendo el método descrito por García-Plazaola y Becerril (2001). Para ello, tanto la medición de fluorescencia, como la toma de muestras de tejido vegetal para pigmentos y antioxidantes, se realizaron al final del experimento, tras un periodo en oscuridad de 12 h, para garantizar condiciones comparables y evitar oscilaciones diurnas (Tausz y cols., 2003).

Además, la eficacia de los procesos de fitorremediación está indudablemente ligada al contenido en metales pesados en los tejidos de la planta: en el caso de la fitoextracción se espera encontrar elevadas concentraciones de metales pesados en la parte aérea de las plantas, mientras que para la fitoestabilización lo ideal es que estos valores sean bajos. De este modo, las muestras de planta eran separadas en raíces y parte aérea, lavadas, y sometidas a 70 ºC durante 48 h para la determinación de su peso seco. Posteriormente, las muestras se homogenizaban y digerían en una mezcla de HNO_3/$HClO_4$ para la determinación de concentración de metales pesados en planta, de acuerdo al método descrito por Zhao y cols. (1994), utilizando un equipo de Espectrometría de Absorción Atómica de llama (Spectra AA-250 plus, Varian, Australia) (Capítulos 5, 6 y 7).

Figura 3.3: Determinación de metales pesados en planta: (a) toma de muestra de material vegetal; (b) espectrómetro de absorción atómica de llama. Fuente: NEIKER-Tecnalia.

3.2.2 Propiedades físico-químicas de suelo

Aunque el pilar fundamental sobre el que se sustenta este trabajo es el análisis de las propiedades microbianas para evaluar la salud del ecosistema edáfico, las propiedades físico-químicas, que se han empleado tradicionalmente, siguen siendo herramientas muy útiles que aportan información complementaria acerca de la salud del suelo. De este modo, se midieron una serie de parámetros físico-químicos como:

- pH, siguiendo método estandarizado (MAPA, 1994), parámetro fundamental, entre otros aspectos, para entender y predecir el comportamiento y movilidad de los metales pesados presentes en el suelo.
- Materia orgánica (MAPA, 1994) y carbono orgánico soluble en agua (Epelde y cols., 2010b) para determinar aspectos relacionados con la fertilidad del suelo.
- Carbono y nitrógeno totales, determinados de acuerdo a los procedimientos descritos en las normas ISO 10694 (1995) y ISO 13878 (1998), respectivamente; amonio, determinado según Nelson (1983); fósforo extraíble, potasio, calcio y magnesio intercambiables, y capacidad de intercambio catiónico, se midieron de acuerdo a métodos estandarizados (MAPA, 1994). Estos parámetros están relacionados con los nutrientes y su disponibilidad.
- Textura (MAPA, 1994) y capacidad de retención hídrica (Richards, 1948): fundamentales para predecir la capacidad de retención y transporte de agua, nutrientes y compuestos químicos.

Por otro lado, tratándose de suelos contaminados con metales pesados, se determinó la concentración total de metales (mediante la digestión del suelo en una mezcla de $HNO_3/HClO_4$), así como la concentración de metales extraíbles en $CaCl_2$ presentes en el suelo, siguiendo los métodos descritos por Zhao y cols. (1994) y Houba y cols. (2000), respectivamente. Así mismo, en los Capítulos 5 y 6, se determinó la concentración de metales solubles en agua, mediante la toma de muestra con Rhizons (Rhizosphere Research Products, B.V.).

3.2.3 Propiedades microbianas con potencial indicador de la salud del suelo

Aunque existen interpretaciones diversas sobre el concepto de calidad o salud del suelo, hay una opinión mayoritaria de que dicho concepto está relacionado con la capacidad del suelo de desempeñar sus funciones. Según una definición operativa formulada por Garbisu y cols. (2011), la calidad/salud del suelo se define como "la capacidad de un suelo para realizar sus procesos y servicios ecosistémicos, a la vez que mantiene los atributos ecosistémicos de relevancia ecológica". Esta definición sitúa al suelo como un ente vivo (aunque no un super-organismo) y dinámico sobre el que se sostiene la funcionalidad del ecosistema edáfico.

Las propiedades físico-químicas por sí solas no son suficientes para determinar el efecto de los contaminantes sobre los organismos y sobre las interacciones de éstos con el ecosistema edáfico. Esto dificulta una valoración completa del impacto de los metales pesados, y de la eficacia de los procesos de remediación, sobre el estado de los procesos y servicios ecosistémicos realizados por el ecosistema edáfico. En este sentido, las propiedades biológicas, y especialmente las microbianas, se presentan como indicadores óptimos de la salud del suelo por su rapidez en respuesta, alta sensibilidad y relevancia ecológica. Además, las propiedades microbianas tienen la ventaja de aportar información que integra las distintas propiedades físicas, químicas y biológicas del suelo. Asimismo, se estima que el 90% del flujo energético del suelo está mediado por las comunidades microbianas (Nannipieri y Badalucco, 2003). .

A continuación se detallan los diversos parámetros microbianos analizados en el presente trabajo:

3.2.3.1 Actividades enzimáticas

Las actividades enzimáticas catalizan reacciones asociadas a procesos de vital importancia en el suelo como son la descomposición de la materia orgánica o la

mineralización y el reciclaje de los nutrientes. Precisamente por su papel en estos procesos, las actividades enzimáticas han sido anunciadas repetidamente como indicadores bioquímicos idóneos del impacto negativo de los metales pesados en el ecosistema edáfico, así como de la eficacia de los procesos de remediación. Las enzimas del suelo pueden proceder de microorganismos, vivos o muertos, raíces y partes residuales de plantas, o micro- y mesofauna. En el suelo, las enzimas pueden encontrarse asociadas a células vivas y viables o presentarse de forma extracelular (no asociadas a células viables), así como estabilizadas en la matriz edáfica o complejadas con materia orgánica y arcillas. Así pues, la actividad enzimática en un suelo es consecuencia de la suma de la actividad de enzimas estabilizadas a largo plazo y de la actividad de las comunidades microbianas viables, estimándose que entre el 40 y el 60% de la actividad enzimática del suelo proviene de enzimas estabilizadas.

En este trabajo se han valorado las siguientes actividades enzimáticas:

- *Actividad β-glucosidasa* (Capítulos 4, 5 y 6): siendo una hidrolasa, esta enzima está implicada en la degradación de la materia orgánica (celulosa y hemicelulosa), produciendo glucosa, por lo que tiene un papel importante en el aporte de energía a los microorganismos. Se determinó siguiendo el método descrito por Dick (1997) y Taylor y cols. (2002).
- *Actividad fosfatasa ácida* (Capítulos 4, 5 y 6): implicada en el reciclaje de nutrientes, ya que facilita el aporte de fosfato disponible para las plantas, mediante la liberación de fosfato proveniente de diversas moléculas que conforman la materia orgánica, como nucleótidos y proteínas. Se determinó siguiendo el método descrito por Dick (1997) y Taylor y cols. (2002).
- *Actividad deshidrogenasa* (Capítulos 5, 6 y 7): incluye diversas enzimas capaces de catalizar reacciones implicadas en la oxidación de la materia orgánica. Estas enzimas reflejan únicamente la actividad de las células viables por lo que, en teoría, solo puede tener lugar en células vivas y no en complejos estabilizados del suelo. Así pues, está considerada como una indicadora de la actividad metabólica oxidativa *in situ* existente en el suelo. Esta actividad enzimática se determinó según la norma ISO 23753-2 (2005).
- *Actividad ureasa* (Capítulos 5 y 6): implicada en el ciclo del nitrógeno, catalizando la descomposición del nitrógeno orgánico (urea) en amonio disponible para las plantas. Se midió según Kandeler y Gerber (1988).

- *Actividad glucosaminidasa* (Capítulos 5 y 6): implicada en el ciclo del nitrógeno, catalizando la degradación de quitina en glucosamina. Se determinó según Parham y Deng (2000).
- *Actividad arilsulfatasa* (Capítulos 5 y 6): implicada en el ciclo del azufre, cataliza la mineralización del azufre orgánico, actuando sobre ésteres orgánicos de azufre y liberando sulfato disponible para las plantas. Se determinó siguiendo el método descrito por Dick (1997) y Taylor y cols. (2002).
- *Actividad FDA* (Capítulos 5 y 6): implicada en la hidrólisis del diacetato de fluoresceína, está considerada como una indicadora de la actividad microbiana total de un suelo. Se determinó según Shaw y Burns (2006).
- *Actividad arginina deaminasa* (Capítulo 5): implicada en el ciclo del nitrógeno, actúa sobre el aminoácido arginina, liberando amonio. Se midió según Acosta-Martínez y Tabatabai (2000).
- *Actividad amidasa* (Capítulo 5): implicada en la degradación de compuestos carbonados y nitrogenados, liberando amonio disponible para las plantas. Se determinó según Kandeler y cols. (1996).

Figura 3.4: Determinación de actividades enzimáticas. Fuente: NEIKER-Tecnalia.

3.2.3.2 Nitrógeno potencialmente mineralizable

Se trata de una medida comúnmente empleada para evaluar la fertilidad de un suelo. En cambio, en suelos contaminados, como es el caso del presente trabajo, resulta un válido indicador del impacto de la contaminación sobre el funcionamiento del suelo. La mineralización del nitrógeno es un proceso por el cual el nitrógeno orgánico, contenido en la materia orgánica principalmente en forma de aminoácidos y proteínas, es

convertido en distintas formas minerales de nitrógeno inorgánico que pueden ser incorporadas por los productores primarios y así completar el ciclo del nitrógeno. El primer paso es llevado a cabo exclusivamente por microorganismos heterótrofos, en el cual el nitrógeno orgánico es convertido en amonio (amonificación). El amonio es poco duradero, ya que enseguida es oxidado, en un segundo paso, a nitrito y nitrato (nitrificación). Así pues, este parámetro está basado en la medición de la cantidad de amonio producido para obtener una estimación de la tasa de mineralización en un suelo. El nitrógeno potencialmente mineralizable se midió en el Capítulo 4, siguiendo el procedimiento descrito por Powers (1980).

3.2.3.3 Contenido de ATP

El contenido de ATP (trifosfato de adenosina) está considerado un índice tanto de actividad como de biomasa microbiana. Al contrario que algunas actividades enzimáticas, no está asociado ni a células muertas, ni adsorbido a partículas de la matriz edáfica, por lo que está correlacionado con la biomasa microbiana viable. De este modo, su valor puede cambiar en función del estado fisiológico de las células, presentándose como un parámetro altamente sensible a cambios y, por tanto, como un buen indicador de la salud del suelo. El contenido de ATP se determinó en el Capítulo 7, según Webster y cols. (1984) y Ciardi y Nannipieri (1990). El procedimiento está descrito en detalle en el Capítulo 7.

3.2.3.4 Respiración (basal e inducida por sustrato)

La respiración del suelo es un proceso clave en el sistema edáfico; como consecuencia de la respiración efectuada por los microorganismos, el CO_2 es liberado a la atmósfera. La tasa de respiración de un suelo puede variar enormemente, ya que los factores que controlan este proceso son muy diversos (humedad, temperatura, cantidad de materia orgánica, etc.); además, es muy sensible a cambios y, por tanto, a fuentes de estrés como la presencia de contaminantes. Debido a su sensibilidad y relevancia ecológica, la tasa de respiración se presenta como un indicador muy útil a la hora de evaluar el impacto de la contaminación por metales pesados, así como la eficacia de los procesos de fitorremediación, sobre la salud del suelo. La respiración basal del suelo se ha medido en los Capítulos 5, 6 y 7, de acuerdo a la norma ISO 16072 (2002).

Por otro lado, la respiración inducida por sustrato se basa en la medición de la tasa de respiración tras la adición de un sustrato fácilmente mineralizable (glucosa

principalmente), a concentración saturante, en un periodo de tiempo breve. Las reacciones metabólicas ante una concentración no limitante de sustrato alcanzan así, teóricamente, la velocidad máxima. De este modo, la tasa de respiración en estas condiciones da una estimación de la biomasa microbiana potencialmente activa. La respiración inducida por sustrato se ha determinado en los Capítulos 5, 6 y 7 siguiendo la norma ISO 17155 (2002).

3.2.3.5 Carbono de la biomasa microbiana

La medida de biomasa microbiana determina la cantidad de carbono contenida en las comunidades microbianas, es decir, el componente vivo de la materia orgánica de un suelo. La biomasa microbiana es un buen indicador de la salud del suelo, pues responde rápidamente a cambios en las propiedades del suelo, así como a distintas perturbaciones o fuentes de estrés, como la contaminación por metales pesados.

En este trabajo, este parámetro se midió en los Capítulos 5, 6 y 7. El procedimiento está basado en el método de fumigación del suelo con cloroformo, según Vance y cols. (1987), al objeto de lisar las membranas celulares para extraer todo el carbono contenido en los organismos vivos. La concentración de carbono orgánico obtenido se compara entonces con la determinada en un control no-fumigado para así calcular la biomasa de la comunidad microbiana.

3.2.3.6 Abundancia de genes totales mediante qPCR

La abundancia de genes específicos puede determinarse mediante PCR cuantitativa a tiempo real. En esta técnica, la acumulación de las copias del gen amplificado es cuantificada al final de cada ciclo "a tiempo real", en base a un patrón de concentración conocida. En el presente trabajo, se determinó la abundancia de genes de bacterias totales y hongos totales, en los Capítulos 4, 6 y 7. Para ello, se emplearon cebadores que amplifican para los genes 16S rRNA o 18S rRNA, que son universalmente conservados en bacterias y hongos, respectivamente. La cuantificación de dichos genes se utilizó como una estimación de la biomasa de las comunidades bacterianas y fúngicas. En el Capítulo 7, además, se calculó la abundancia de genes de arqueas (mediante 16S rRNA). El método, así como las características de los cebadores, están descritos en detalle en el Capítulo 7.

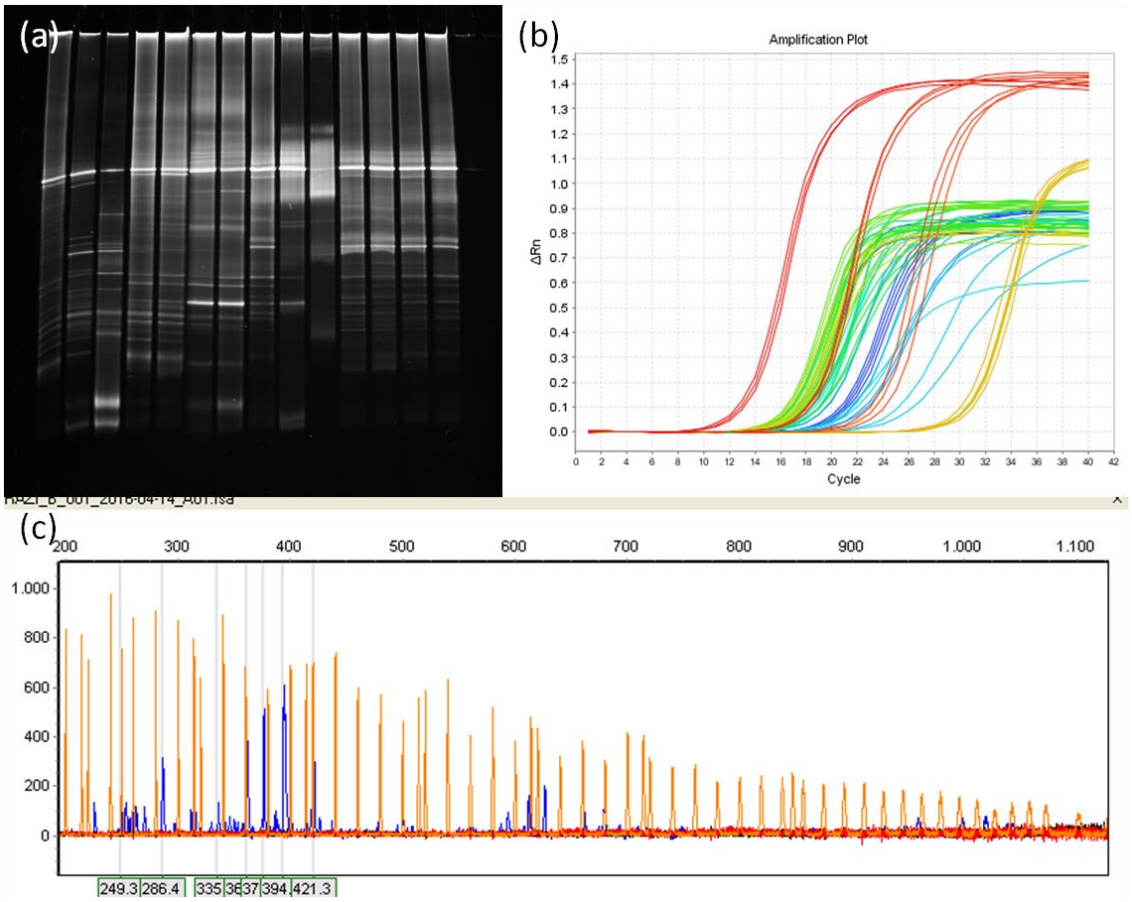


Figura 3.5: (a) Gel de DGGE; (b) gráfica de amplificación obtenido por qPCR; (c) electroferograma obtenido mediante ARISA. Fuente: NEIKER-Tecnalia.

3.2.3.7 Perfiles genéticos a nivel de comunidad (PCR-DGGE)

La técnica de electroforesis en gel de gradiente desnaturalizante se fundamenta en la separación de fragmentos de DNA de la misma extensión (previamente amplificados por PCR) en base a su composición. Para ello, los amplicones se hacen pasar por un gel de acrilamida con un gradiente desnaturalizante (mezcla de urea y formamida). Debido a la variedad de su composición nucleotídica, los fragmentos tienen distintas propiedades, por lo que migran de diferente forma en el gel. De esta manera, cada suelo muestra un patrón de bandas (una "huella" filogenética o perfil de comunidad), en el que cada banda representa un filotipo distinto y el número de bandas puede considerarse como una estimación de riqueza de los filotipos más abundantes de la comunidad microbiana. Además, en función de los cebadores empleados (genes universales vs. genes específicos funcionales), los patrones de bandas obtenidos pueden ser traducidos como estimaciones de diversidad estructural o diversidad funcional. Al ser una técnica que proporciona una imagen o perfil general de las comunidades microbianas de un suelo, resulta muy útil para detectar cambios en la estructura de las comunidades a lo

largo del tiempo o a consecuencia de perturbaciones ambientales, tratamientos, etc. Una de las principales ventajas de este tipo de técnicas moleculares es su independencia respecto al cultivo de los microorganismos. La técnica de PCR-DGGE ha sido utilizada en el Capítulo 7 para la obtención del perfil genético estructural de las comunidades de bacterias, hongos y arqueas, así como para el análisis de perfiles funcionales (degradadores de quitina y nitrito reductasa). Esta técnica está basada en el procedimiento de Epelde y cols. (2012a) y descrita con más detalle en el Capítulo 7.

3.2.3.8 Perfiles genéticos a nivel de comunidad (ARISA)

Esta técnica se basa en la variabilidad de la secuencia del espaciador transcribible interno (ITS), que es una región ubicada entre las subunidades grande y pequeña de los genes universalmente conservados de rRNA, y cuya composición y extensión es muy variable entre los organismos. Para ello, se utilizan unos cebadores, marcados con fluorescencia, que amplifican esta región ITS. De este modo, los distintos amplicones obtenidos son analizados mediante un electroferograma, en el que cada pico representa un fragmento con una extensión distinta, es decir, un filotipo distinto. Este análisis proporciona un patrón de picos o huella genética que puede ser interpretado como un perfil específico a nivel de comunidad y, por tanto, como un indicador de diversidad estructural de las comunidades microbianas de un suelo. Esta técnica es empleada, en los Capítulos 5 y 6, para obtención de perfiles de comunidades bacterianas y fúngicas, según Cardinale y cols. (2004) y Ranjard y cols. (2000), respectivamente.

3.2.3.9 Perfiles fisiológicos a nivel de comunidad (Biolog EcoPlates™)

Las Biolog EcoPlates™ proporcionan un patrón catabólico de utilización de fuentes de carbono de las comunidades microbianas de un suelo determinado. Esta información sirve como indicador de la diversidad funcional de las comunidades microbianas y, por tanto, de la salud del suelo. Las placas constan de 31 pocillos con diversos sustratos de carbono (carbohidratos, ácidos carboxílicos, aminas/amidas, aminoácidos, polímeros) y contienen tetrazolio que, en caso de reacción catabólica, desarrolla un color morado. La intensidad y el patrón de la formación de esa coloración morada, entre los 31 pocillos, proporcionan una "huella" fisiológica representativa de la diversidad funcional microbiana y, por lo tanto, puede ser empleada para comparar el efecto de distintos tratamientos experimentales sobre las comunidades microbianas de un suelo. Los

perfiles fisiológicos con Biolog EcoPlates™ fueron determinados en los Capítulos 6 y 7, según Epelde y cols. (2008a) e Insam (1997).

3.2.3.10 Ensayo de supresividad (test del anillo de agar)

El método del anillo de agar proporciona una estimación de la supresividad de un suelo. Los suelos supresivos son suelos en los que la incidencia y severidad de plagas se mantiene baja, a pesar de la presencia de un patógeno, una planta hospedadora susceptible y condiciones climáticas favorables para el desarrollo de plagas (Janvier y cols., 2007). Esta capacidad está considerada como un atributo muy importante para un suelo saludable, fuertemente ligada al estado (actividad, biomasa y diversidad) de sus comunidades microbianas y, por tanto, altamente sensible a cambios o perturbaciones.

Figura 3.6: Determinación de supresividad con el test del anillo de agar. Fuente: NEIKER-Tecnalia.

La técnica se basa en la utilización y crecimiento de un hongo patógeno, *Rhizoctonia solanum*, sobre un anillo que contiene una muestra de suelo enriquecido con agar. El crecimiento radial de dicho hongo sobre el anillo será mayor o menor en

función de la capacidad de ese suelo de evitar por difusión el desarrollo del patógeno. Esta técnica es empleada en los Capítulos 5, 6 y 7, siguiendo el procedimiento descrito por Grünwald y cols. (1997) y Nuñez-Zofío y cols. (2012).

3.2.3.11 Ensayo de estabilidad (resiliencia y resistencia)

Este método tiene la finalidad de obtener una estimación de la estabilidad de un suelo, es decir, su capacidad para mantener su estructura y funcionamiento ante una perturbación. La estabilidad de un suelo se refleja en su *resistencia*, la capacidad de ese suelo de soportar una perturbación y mantener sus funciones, y su *resiliencia*, la capacidad del suelo de recuperarse tras la perturbación y volver a su estado inicial. Por consiguiente, en este ensayo, se aplica una perturbación o fuente de estrés y se observa la evolución a lo largo del tiempo de una serie de parámetros. En el presente trabajo la perturbación consistió en aplicar calor (42 ºC) durante 24 h. Las estimaciones de los índices de resistencia y resiliencia se calculan a tiempo 0 (inmediatamente después de la aplicación de la perturbación) y al final del ensayo (15 semanas), mediante la determinación de la actividad deshidrogenasa (ISO 23753-2, 2005) y la tasa potencial de nitrificación (ISO 15685, 2004). El ensayo de estabilidad es empleado y descrito en detalle en el Capítulo 7.

3.2.4. Rasgos de promoción del crecimiento vegetal en bacterias endófitas

Una vez aisladas las cepas bacterianas endófitas de los Capítulos 5 y 6, éstas se analizaron para una serie de características que, de acuerdo a varios autores (Arshad y cols., 2007; Glick y cols., 2007; Rajkumar y cols., 2009; Ma y cols., 2011; Zhang y cols., 2011), son las responsables de promover el crecimiento de las plantas en las que se hospedan. Estas características pueden fomentar el crecimiento de las plantas directamente, mediante la producción de fitohormonas o facilitando la toma de nutrientes, o indirectamente, participando en vías metabólicas que inducen cambios a nivel fisiológico. Entre estos atributos se encuentran:

- *Actividad ACC deaminasa*: la enzima ACC-deaminasa utiliza como sustrato el ácido aminociclopropanocarboxílico (ACC), catalizando una reacción de la cual se obtiene amonio y α-cetobutarato como subproductos. El ACC es un precursor del etileno que en las plantas está implicado en la respuesta al estrés, desencadenando una serie de efectos tales como senescencia de las hojas y flores, paralización del crecimiento radicular, etc. En este sentido, la actividad

ACC-deaminasa en las plantas desvía la utilización del ACC hacia la producción amonio y α-cetobutarato (Figura 3.7), reduciendo así la producción de etileno y favoreciendo la atenuación de los efectos inhibitorios de la respuesta de las plantas al estrés. Este hecho tiene una serie de implicaciones a nivel fisiológico que permiten a las plantas poder llevar a cabo su desarrollo a pesar de la existencia de fuentes de estrés como los metales pesados. Según Glick y cols. (2007), esta enzima supone uno de los mecanismos principales del que disponen las bacterias promotoras de crecimiento vegetal para favorecer el crecimiento de las plantas. Además, la ACC-deaminasa está presente en una amplia variedad de cepas de bacterias y hongos, y es relativamente común entre los microorganismos del suelo. Por tanto, esta característica está considerada como un rasgo importante a analizar y valorar en la búsqueda de bacterias con potencial promotor del crecimiento vegetal (PGP) aisladas del suelo, rizosfera o interior de plantas. Así pues, en este trabajo, solamente las bacterias con actividad ACC-deaminasa fueron tenidas en cuenta y estudiadas para el resto de rasgos PGP, descartándose las cepas que no presentaban esta actividad. Para ello se siguió el procedimiento según Penrose y Glick (2003).

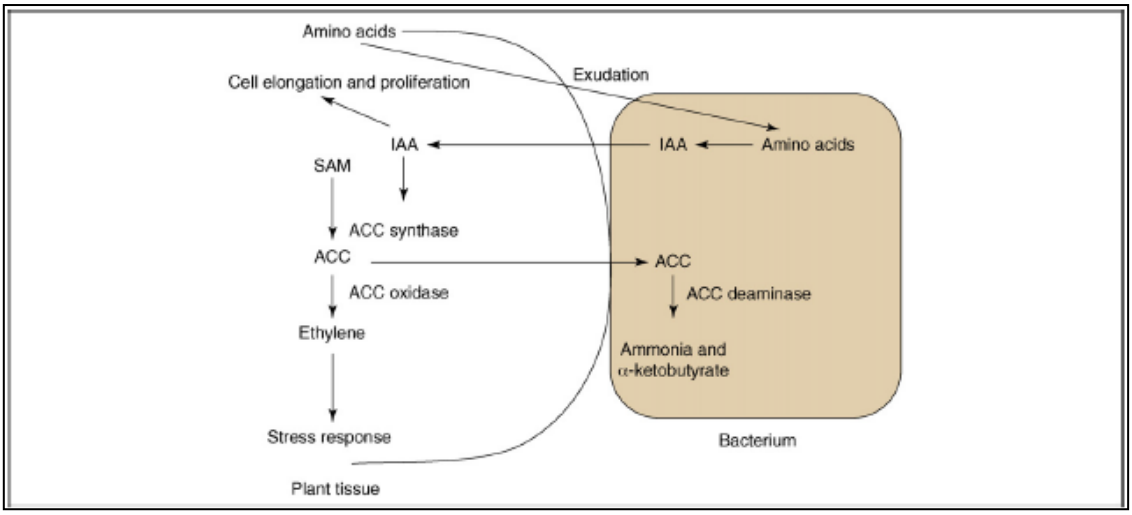


Figura 3.7: Síntesis y rutas del ACC y IAA. Fuente: Arshad y cols. (2007).

- *Producción de IAA*: el ácido indol-3-acético (IAA) es una auxina que, al igual que otras fitohormonas como las citoquininas y giberelinas, tiene la capacidad de estimular la germinación, el crecimiento y la reproducción, así como proteger a las plantas frente a fuentes de estrés biótico y abiótico. El IAA, también llamado

"hormona de crecimiento", es producido por las plantas y transportado a las raíces; está asociado a la elongación y división celular, contribuyendo al crecimiento y al desarrollo del sistema defensivo de las plantas (Ma y cols., 2011). Sus rutas de síntesis parten en su mayoría del L-triptófano, por lo que, en respuesta al triptófano y otras pequeñas moléculas presentes en los exudados de las raíces, ciertas bacterias asociadas a las plantas pueden sintetizar y secretar IAA al medio externo, parte del cual es captado por las plantas. Este IAA, junto con el IAA endógeno, puede estimular la proliferación y la elongación celular en las plantas a través de factores de respuesta a auxinas (ARF). Las rutas del IAA y el ACC están interrelacionadas, tal y como puede verse en la Figura 3.7, ya que el IAA también estimula la actividad de la ACC-sintetasa, la cual cataliza la formación de ACC. El ACC, a su vez, es el precursor inmediato del etileno, el cual reprime la síntesis de los ARF e inhibe el transporte del IAA en plantas. De esta manera, queda demostrado el efecto antagónico del etileno contra el IAA (esquematizado en la Figura 3.8) para limitar el crecimiento y prevenir el gigantismo en plantas. La producción de IAA se determinó según Becerra-Castro y cols. (2011).

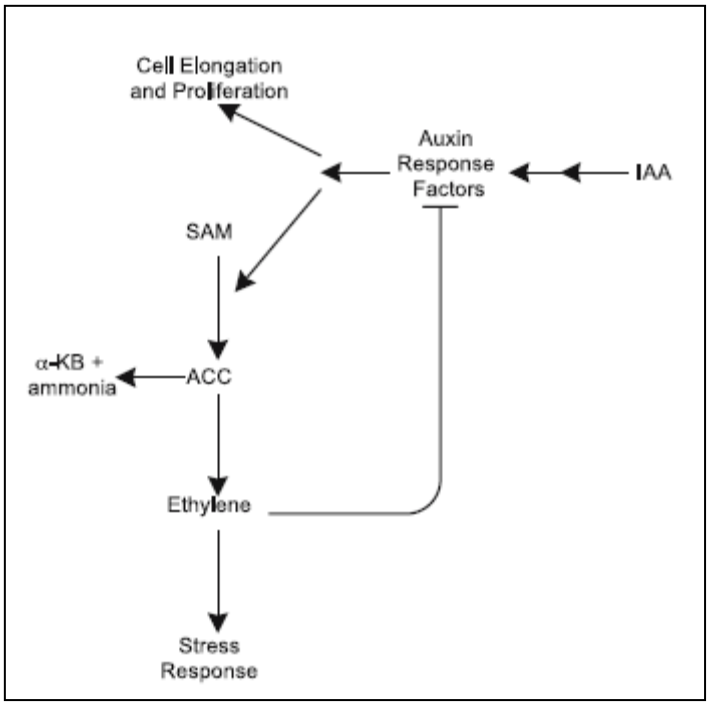


Figura 3.8: Representación esquemática del efecto antagónico del etileno sobre las ARF. Fuente: Glick y cols. (2007).

- *Producción de sideróforos*: los sideróforos son quelantes de bajo peso molecular con gran afinidad por el Fe^{3+} con el que forman complejos sideróforo-Fe^{3+}. Una vez formados, estos complejos son captados por las membranas bacterianas donde el Fe^{3+} es reducido a Fe^{2+} y liberado al interior de las células para ser aprovechado. La producción de estos quelantes es importante, ya que el hierro es un nutriente esencial para la vida, pero que en el ambiente suele encontrarse de forma insoluble y poco disponible para los microorganismos (en forma de hidróxidos y oxihidróxidos). De esta forma, las bacterias endófitas con capacidad para producir sideróforos pueden incrementar la disponibilidad de hierro para las plantas, mejorando así su estado nutricional. Pero además de hierro, los sideróforos también pueden formar complejos estables con otros metales como Cd, Pb, Zn, Al y Cu (Rajkumar y cols., 2010), lo que resulta de gran interés en procesos de fitoextracción en los que la reducida biodisponibilidad de los metales supone una limitación. La determinación de la producción de sideróforos está basada en el método universal CAS (cromo-azurol-S) descrito por Schwyn y Neilands (1987).
- *Solubilización de fosfato inorgánico*: el fósforo es un macronutriente esencial para el crecimiento y desarrollo biológicos que, al encontrarse normalmente en formas no disponibles para las plantas, resulta ser el más limitante para la producción de biomasa en los ecosistemas naturales. Además, en suelos contaminados, las elevadas concentraciones de metales interfieren con la incorporación de fósforo, dificultando así el crecimiento de las plantas. En este sentido, las PGPB tolerantes a los metales y con capacidad para solubilizar fosfato pueden convertir fosfato insoluble en formas disponibles para las plantas, mediante procesos de acidificación, quelación o liberación de ácidos orgánicos (Ma y cols., 2011). La capacidad para solubilizar fosfato inorgánico se valoró según el procedimiento descrito por Nautiyal (1999).

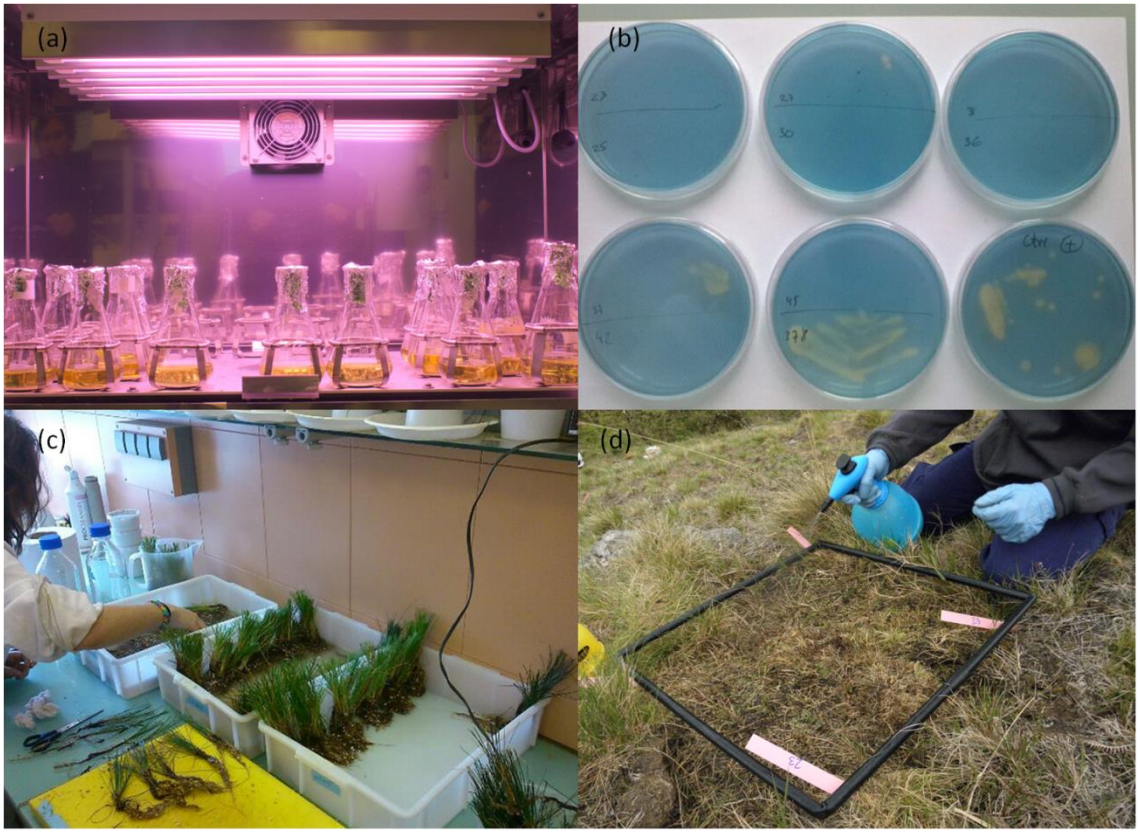


Figura 3.9: Empleo de endófitas en ensayos de fitorremediación: (a) crecimiento de los cultivos bacterianos; (b) determinación de la capacidad de producción de sideróforos; (c) inoculación de plantas por inmersión en suspensión bacteriana; (d) inoculación por "spray" de suspensión bacteriana. Fuente: NEIKER-Tecnalia.

Así mismo, se efectuó una caracterización fenotípica de las cepas bacterianas con el objetivo de determinar su valencia ecológica, de cara a seleccionar las más idóneas para procesos de fitorremediación en ambientes mineros.

- *Tolerancia a metales*: el éxito de un proceso fitorremediador depende en gran medida de la tolerancia a los metales de las plantas empleadas; análogamente, es fundamental que las bacterias asociadas a esas plantas también presenten resistencia a elevadas concentraciones de metales. La tolerancia de las cepas bacterianas se determinó según Long y cols. (2011).
- *Tolerancia a salinidad*: si bien es cierto que una salinidad elevada representa un estrés abiótico importante para el crecimiento de la mayoría de plantas, este factor no parece ser un problema en el suelo de la mina objeto de este estudio. Sin embargo, según Rashid y cols. (2012), en el caso de bacterias que tienen que vivir y multiplicarse en el interior de las plantas (bacterias endófitas), donde el medio contiene una fuerza iónica relativamente elevada, una tolerancia a

concentraciones más o menos elevadas puede ser una característica deseable. Por ello, la tolerancia a la salinidad de las cepas fue evaluada según Rashid y cols. (2012).

- *Caracterización fenotípica con placas GEN III*: las placas GEN III proporcionan una huella fenotípica de las cepas bacterianas mediante los 94 tests incorporados en cada placa. De esta forma, 71 de los 96 pocillos contienen sustratos de las principales fuentes de carbono, obteniéndose un patrón de utilización de fuentes de carbono de las cepas estudiadas. Por otro lado, 23 pocillos aportan información acerca de la sensibilidad y propiedades fisiológicas, determinando la tolerancia de las cepas a distintos pH, salinidad, antibióticos y otros inhibidores. Toda esta información proporciona una estimación de la viabilidad ambiental de las cepas bacterianas, con el objetivo de determinar cuáles tienen más posibilidades de supervivencia y éxito en el ecosistema.

3.3 Tratamiento de datos

De forma general, el efecto de los tratamientos sobre los parámetros físico-químicos y microbianos del suelo, así como sobre los parámetros de planta, fueron evaluados mediante análisis de la varianza (ANOVA) de una, dos o tres vías, en función del número de factores que componían los tratamientos. En ANOVA de una vía, y en caso de existir diferencias significativas, la significancia de las diferencias entre las medias correspondientes a los tratamientos se determinó con el test Tukey-Kramer (Capítulo 4). En los ensayos en los que los tratamientos estaban compuestos por más de un factor, el efecto de cada factor por separado, así como el efecto de sus interacciones, se evaluó mediante ANOVA de dos (Capítulos 5 y 7) o tres vías (Capítulo 6); en caso de que la ANOVA indicara que un factor, o una interacción, tuviera un efecto significativo, las diferencias entre los factores, o niveles de los factores, se analizaron mediante el test de Fisher (LSD). En el Capítulo 7, se evaluó el efecto del momento de muestreo mediante ANOVA de medidas repetidas.

Además, se realizaron análisis multivariantes con el objetivo de explorar la relación entre los factores experimentales y los parámetros de la salud del suelo. Para ello, en los Capítulos 5 y 6, se determinó la influencia de los tratamientos sobre los parámetros de suelo y planta analizados, mediante análisis de redundancia (RDA) y análisis de partición de la varianza. En el Capítulo 7, se realizó un análisis de componentes principales (PCA) a partir de los valores de absorbancia obtenidos en cada

tratamiento con las placas Biolog EcoPlates™. En el Capítulo 4, el efecto de la contaminación reiterada, a lo largo del tiempo, sobre las propiedades microbianas se estimó, primeramente, mediante modelos aditivos generalizados (GAM), con el objetivo de obtener la tendencia temporal de cada parámetro, por separado, para cada metal pesado; y, posteriormente, se aplicó un análisis de curva de respuesta principal (PRC) para evaluar la respuesta temporal del conjunto de las propiedades microbianas del suelo, para cada metal pesado, tras los distintos episodios contaminantes.

Para simplificar y facilitar la interpretación de los resultados, se estimaron índices o categorías de nivel superior, calculados a partir de los datos de los diferentes parámetros analizados, que aportaban medidas integradoras relativas a la salud del suelo, o a los atributos y servicios ecosistémicos aportados por el mismo. Para ello, primeramente, los valores medidos fueron transformados en porcentajes, donde el valor del suelo de referencia (suelo control) era de 100% y los valores correspondientes a los tratamientos eran porcentajes respecto al control.

A continuación, se calculó el Índice de Calidad del Suelo (SQI), usando la fórmula de Bloem y cols. (2006):

$$SQI = 10^{\log m - \frac{\sum_{i=1}^{n} |\log m - \log n_i|}{n}}$$

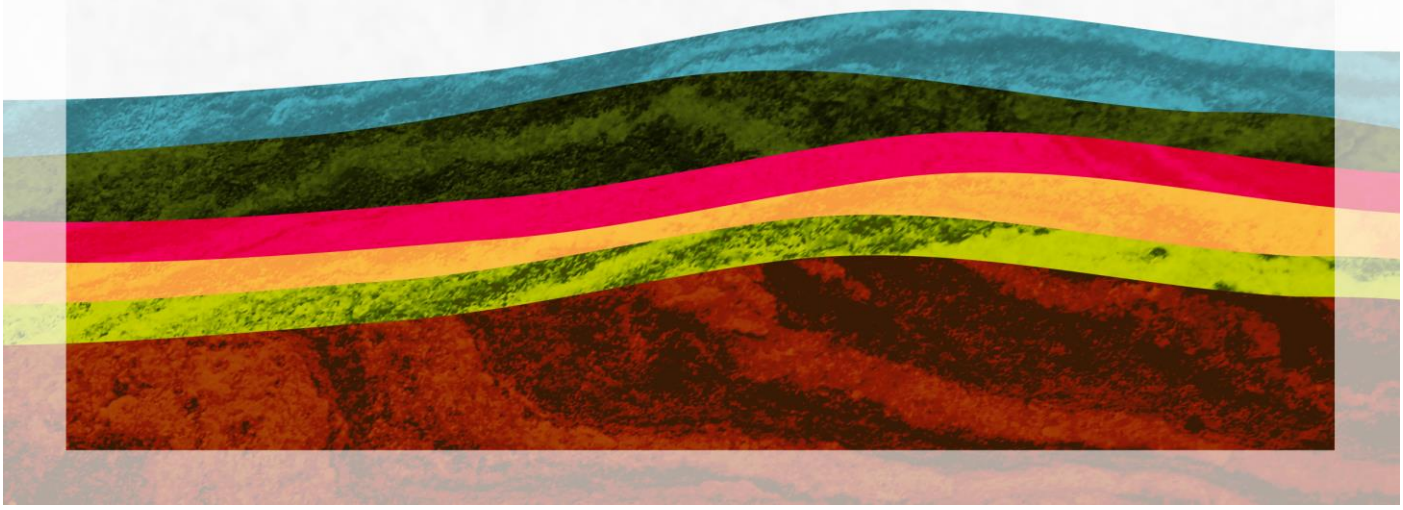
donde *m* es el valor de referencia (suelo control) y *n* son los valores medidos, todos expresados como porcentajes.

Por otro lado, la estimación de categorías de un nivel superior (CNS), efectuada en los Capítulos 5, 6 y 7, en forma de atributo ecológico o servicio ecosistémico, se realizó aplicando la siguiente fórmula, modificada a partir del índice T-SQI propuesto por Mijangos y cols. (2010):

$$CNS = 10^{\log m + \frac{\sum_{i=1}^{n} (\log n_i - \log m)}{n}}$$

donde *m* es el valor de referencia (suelo control) y *n* corresponde a los valores medidos para cada parámetro. El hecho de que el cálculo de categorías de nivel superior esté basado en el índice T-SQI resulta muy apropiado para suelos que han sido intencionadamente tratados con el objetivo de incrementar ciertos parámetros y recuperar la salud del suelo, como es el caso de los tratamientos en los Capítulos 5-7. Esto se debe a que dicha fórmula tiene en consideración no solo la magnitud del cambio, sino también la dirección del mismo (incremento/disminución).

04 IMPACT OF REPEATED SINGLE-METAL AND MULTI-METAL POLLUTION EVENTS ON SOIL QUALITY



4. IMPACT OF REPEATED SINGLE-METAL AND MULTI-METAL POLLUTION EVENTS ON SOIL QUALITY

Burges A, Epelde L, Garbisu C, 2015. Impact of repeated single-metal and multi-metal pollution events on soil quality. ***Chemosphere*** *120, 8-15.*

Abstract

Most frequently, soil metal pollution results from the occurrence of repeated single-metal and, above all, multi-metal pollution events, with concomitant adverse consequences for soil quality. Therefore, in this study, we evaluated the impact of repeated single-metal and multi-metal (Cd, Pb, Cu, Zn) pollution events on soil quality, as reflected by the values of a variety of soil microbial parameters with potential as bioindicators of soil functioning. Specifically, parameters of microbial activity (potentially mineralizable nitrogen, β-glucosidase and acid phosphatase activity) and biomass (fungal and bacterial gene abundance by RT-qPCR) were determined, in the artificially metal-polluted soil samples, at regular intervals over a period of 26 weeks. Similarly, we studied the evolution over time of $CaCl_2$-extractable metal fractions, in order to estimate metal bioavailability in soil. Different metals showed different values of bioavailability and relative bioavailability ($[metal]_{bio}/[metal]_{tot}$) in soil throughout the experiment, under both repeated single-metal and multi-metal pollution events. Both repeated Zn-pollution and multi-metal pollution events led to a significant reduction in the values of acid phosphatase activity, and bacterial and fungal gene abundance, reflecting the negative impact of these repeated events on soil microbial activity and biomass, and, hence, soil quality.

4.1 Introduction

Soil is a heterogeneous, dynamic, complex living ecosystem that represents a unique balance between physical, chemical and biological properties and whose condition is vital to the proper function of terrestrial ecosystems (Doran and Parkin, 1996). Regrettably, due to a variety of anthropogenic activities (including smelting, mining and agricultural activities), soil pollution with toxic metals is currently an environmental problem of great concern worldwide (Gómez-Sagasti et al., 2012). Indeed, metal pollution is negatively affecting soil quality at a global scale with deleterious effects on the valuable ecosystem services provided by the soil ecosystem (Jeffery et al., 2010).

Soil quality has been defined as "the capacity of soil to perform its functions" (Doran and Parkin, 1996) and, more recently, as "the capacity of a given soil to sustainably perform its ecological processes, functions and ecosystem services at a level similar to that of a reference soil, without causing an adverse impact on the proper functioning of surrounding ecosystems or human health" (Garbisu et al., 2011). Accordingly, it is imperative to have a reliable set of indicators of soil quality which must provide information on the impact of disturbances on soil functioning (Karlen et al., 1997). Traditionally, soil physicochemical parameters have been used as indicators of soil quality; nonetheless, microbial parameters are increasingly being used as bioindicators of soil quality owing to their rapid response, sensitivity, ecological relevance, and capacity to provide information that integrates many environmental factors (Epelde et al., 2009a). Indeed, microbial communities play a key role in many soil processes (*e.g.*, organic matter decomposition, nutrient cycling) and the delivery of essential soil ecosystem services (Jeffery et al., 2010). After all, soil microorganisms have intimate relations with their surroundings due to their high surface to volume ratio, and are, to a great extent, responsible for the health of the soil ecosystem (Nannipieri et al., 2003).

On the other hand, the capacity of a given soil to recover from disturbances (its resilience) can be assessed by monitoring soil microbial activities (Nannipieri et al., 2003). Hence, microbial parameters reflecting the biomass, activity and diversity of soil microbial communities have been frequently used as bioindicators not only of the impact of metals on soil quality (Rusk et al., 2004; Kao et al., 2006) but also to evaluate the effectiveness of soil metal remediation processes (Epelde et al., 2009b; 2009a).

Yet, many of these studies were carried out in soils artificially polluted through a single-metal pollution event. By contrast, most frequently, soil metal pollution results from the occurrence of repeated single-metal and, above all, multi-metal pollution events (Mertens et al., 2006). On the other hand, van Bruggen and Semenov (2000) emphasized the importance of monitoring soil microbial responses at regular intervals after the application of stresses.

The aim of this work was to assess the impact of repeated single-metal and multi-metal (Cd, Pb, Cu, Zn) pollution events on soil quality, through the study of the evolution over time of a variety of soil microbial parameters with potential as bioindicators of soil functioning. Similarly, the evolution over time of $CaCl_2$-extractable

metal (Cd, Pb, Cu, Zn) fractions in the polluted soil samples was also determined as an estimation of metal bioavailability and, hence, toxicity.

4.2 Materials and methods

4.2.1 Soil characterization and experimental design

A microcosm study was carried out with soil collected from the upper 20 cm layer of a natural grassland located in Derio (Basque Country, northern Spain). Immediately after collection, the soil was air-dried at 25 °C for 24 h and sieved to <2 mm. Soil physicochemical parameters were determined according to standard methods (MAPA, 1994). The soil was a clay loam, with a water holding capacity (WHC) of 71.5%, a pH of 5.2, an organic matter (OM) content of 4.12%, a total nitrogen (N) content of 0.23%, a C/N ratio of 10.4, a phosphorus (P) content of 26.4 mg kg^{-1}, and an electrical conductivity of 0.08 dS m^{-1}.

Prior to metal treatment application, the soil was preconditioned in 0.5 L plastic pots (with holes to allow aeration) for 2 weeks under the following experimental conditions: darkness, 20 °C and 60% WHC. Then, soil portions of 250 g of dry weight (DW) soil were individually polluted (by mechanical mixing in plastic trays) with Cd (36 mg kg^{-1} DW) as $CdCl_2$, Pb (660 mg kg^{-1} DW) as $PbCl_2$, Cu (500 mg kg^{-1} DW) as CuCl, Zn (1680 mg kg^{-1} DW) as $ZnCl_2$, and a combination of all of them (*i.e.*, multi-metal treatment): 36 Cd + 660 Pb + 500 Cu + 1680 Zn (in mg kg^{-1} DW). These four metals were chosen here due to their environmental relevance; on the other hand, the choice of metal concentrations was based on the decision of doubling the Reference Critical Values reported for the protection of ecosystems in the Basque Country (*i.e.*, 18, 330, 250 and 830 mg kg^{-1} for Cd, Pb, Cu and Zn, respectively) (IHOBE, 1998). The same soil was used as control (without metal application). All six treatments (*i.e.*, Cd, Pb, Cu, Zn, multi-metal, control) were run in triplicate.

Then, soil samples were incubated in 0.5 L plastic pots for 26 weeks under the same experimental conditions described above. Throughout the 26-week incubation period, WHC was held constant at 60% by adding distilled water. After 9 and 18 weeks of incubation, a second and third metal pollution event, respectively, was applied to all soil samples using the same treatments mentioned above. Then, the impact of these repeated (3 times: at weeks 0, 9 and 18) single-metal and multi-metal pollution events on soil quality was assessed through the study of the evolution over time of a variety of soil microbial parameters with potential as bioindicators of soil functioning.

4.2.2 Soil metal bioavailability

For the determination of soil metal bioavailability, the soil was sampled 4 weeks after each one of the three pollution events. $CaCl_2$-extractable (0.01 M) metal fractions in soil, as an indicator of metal bioavailability (Novozamsky et al., 1992; Houba et al., 2000), were determined as described by Houba et al. (2000). Cadmium, Pb, Cu and Zn concentrations in the extracts were analysed by flame atomic absorption spectrometry (Varian).

4.2.3 Soil microbial parameters

For the determination of soil microbial parameters, 10 g of FW (fresh weigh) soil was sampled, from the experimental pots, 1 day, 1 week, 4 weeks and 8 weeks after each one of the three pollution events (*i.e.*, 3 pollution events x 4 sampling times = a total of 12 samples). Soil microbial parameters were also determined twice (after 1 and 2 weeks) during the 2-week preconditioning period, confirming their stability before the soil was distributed into portions prior to treatment application (data not shown). Before each sampling, soil was mixed thoroughly with a spatula in order to ensure the homogeneity of the sample.

β-glucosidase and acid phosphatase activities were determined according to Dick et al. (1997) and Taylor et al. (2002). Potentially mineralizable nitrogen, N_{min}, an indicator of the capacity of the soil to supply plant-available nitrogen, was measured as described by Powers (Powers, 1980).

Soil samples for DNA analysis were stored fresh at -20 °C. DNA was extracted from soil samples (0.25 g of DW soil) using Power Soil™ DNA Isolation Kit (MO BIO Laboratories, California, USA) according to the manufacturer's specifications. Real-time qPCR (RT-qPCR) was carried out for measurements of bacterial and fungal gene copy abundance as described in Epelde et al. (2014b). The primers used to assess 16S rRNA gene fragments for total bacteria were Ba519F and Ba907R; the primers used to assess 18S rRNA gene fragments for total fungi were Fung5f and FF390r (Lueders et al., 2004a; 2004b).

4.2.4 Statistical analysis

Differences among treatments for values of $CaCl_2$-extractable metal concentrations and soil microbial parameters were analysed with one-way ANOVA using Microsoft Stat

View Software (SAS Institute, 1998). Tukey-Kramer test was used to establish the significance of the differences among means. The Soil Quality Index (SQI) was determined from the values of all soil microbial parameters according to Bloem et al. (2006):

$$SQI = 10^{\log m - \frac{\sum_{i=1}^{n} |\log m - \log n_i|}{n}}$$

where *m* is the reference (mean value of control non-polluted soil, set to 100%) and *n* are the measured values as percentages of the reference.

Multivariate analyses were applied to explore the relationships between experimental factors and response variables using Canoco 5 (ter Braak and Šmilauer, 2012). First, the temporal trends of the response variables were assessed by fitting them to Generalized Additive Models (GAMs). Generalized additive models combine the ability to explore many nonparametric relationships simultaneously with the distributional flexibility of generalized linear models, being useful in finding predictor-response relationships in many kinds of data (Hastie and Tibshirani, 1986). Then, a Principal Response Curve (PRC) Analysis was applied to our data. In addition, the statistical significance of the effects due to the two main factors (metal treatment and time) was tested by means of variation partitioning analyses. Data were centered and standardized before analyses were performed; moreover, to obtain P-values, 999 permutations of residuals under a full model were performed.

4.3 Results

4.3.1 Soil metal bioavailability

In the control (non-polluted) soil, $CaCl_2$-extractable concentrations for Cd, Pb and Cu (but not for Zn) were below detection limit (Table 4.1). In both single-metal and multi-metal polluted soils, values of $CaCl_2$-extractable metal concentration increased progressively with successive pollution events for all four metals. Moreover, in single-metal treatments, values of "relative metal bioavailability" for all four metals (*i.e.*, bioavailable metal concentration divided by total metal concentration; $[metal]_{bio}/[metal]_{tot}$), showed a slight tendency to increase throughout the 26-week experimental period: values were within the range of 32.5-37.9% for Cd, 1.4-2.0% for Pb, 1.4-3.2% for Cu and 7.1-20.4% for Zn. After the third pollution event, values of

relative bioavailability for Pb, Cu and Zn were up to 2-fold, 2.3-fold and 2.9-fold higher, respectively, than after the first pollution event (Table 4.1).

Table 4.1. Effect of treatments on $CaCl_2$-extractable metal concentrations (mg kg^{-1} DW) in soil 4 weeks after each pollution event (average value ± standard error; n = 3). Values followed by different letters are significantly different ($p<0.05$) according to Tukey-Kramer test (single-metal *versus* multi-metal). Values of relative metal bioavailability ($[metal]_{bio}/[metal]_{tot}$), as percentages, are shown within brackets.

	Cd		Pb		Cu		Zn	
	First pollution event							
Control	<0.1		<0.1		<0.025		6.2 ± 2.4	
Cd	11.7 ± 0.4[a]	(32.5%)						
Pb			6.8 ± 1.6[a]	(1.0%)				
Cu					7.3 ± 1.3[a]	(1.4%)		
Zn							119.7 ± 14.5[a]	(7.1%)
Multi-metal	16.3 ± 1.0[b]	(44.4%)	6.3 ± 0.2[a]	(1.0%)	7.6 ± 0.4[a]	(1.5%)	182.8 ± 8.0[b]	(10.9%)
	Second pollution event							
Control	<0.1		<0.1		<0.025		7.4 ± 2.8	
Cd	24.3 ± 0.8[a]	(33.7%)						
Pb			19.3 ± 3.4[a]	(1.4%)				
Cu					31.5 ± 9.2[a]	(3.1%)		
Zn							351.4 ± 58.9[a]	(10.4%)
Multi-metal	38.4 ± 1.1[b]	(53.3%)	22.0 ± 6.7[a]	(1.6%)	56.1 ± 6.7[b]	(5.6%)	387.3 ± 62.3[a]	(11.5%)
	Third pollution event							
Control	<0.1		<0.1		<0.025		7.3 ± 1.2	
Cd	41.4 ± 0.8[a]	(37.9%)						
Pb			38.9 ± 5.0[a]	(2.0%)				
Cu					49.6 ± 7.1[a]	(3.2%)		
Zn							1033.1 ± 14.9[a]	(20.4%)
Multi-metal	68.1 ± 5.2[b]	(63.0%)	82.3 ± 14.4[b]	(4.1%)	158.5 ± 28.3[b]	(10.5%)	1316.0 ± 68.1[b]	(26.1%)

Interestingly, when the soil was polluted with a mixture of the four metals (multi-metal treatment), values of bioavailable concentration in soil for each metal were in many cases higher than those observed under single-metal treatments. These differences in metal bioavailability between single-metal and multi-metal polluted soils were more pronounced as total metal concentration in soil increased throughout the experiment, as a result of additional (second and third) pollution events. After the third pollution event, values of relative metal bioavailability in the multi-metal polluted soil were 4.1-, 7.0- and 2.4-fold higher for Pb, Cu and Zn, respectively, as compared to the first pollution event.

4.3.2 Soil microbial parameters

The impact of repeated single-metal and multi-metal pollution events on soil microbial parameters at the end of the experiment is summarized in Table 4.2. Regarding microbial activity, we assessed the effect of repeated pollution events on potentially mineralizable nitrogen (N_{min}), acid phosphatase and β-glucosidase activity. Compared to control soil, Cu, Zn and multi-metal treatments significantly decreased values of acid phosphatase activity (lowest values were observed under the multi-metal treatment). Regarding β-glucosidase activity, Zn and Pb treatments led to significantly lower and higher values, respectively, compared to control soil. Finally, no significant differences were observed for values of N_{min} among the studied treatments.

Trends over time of these microbial parameters were analysed by General Additive Models (GAMs), performed with each microbial parameter as a response variable and time as a covariate (Figure 4.1). GAMs for acid phosphatase (Figure 4.1a) and β-glucosidase (Figure 4.1b) activities confirmed the aforementioned effects: an inhibition of acid phosphatase activity in Cu, Zn and multi-metal treatments; an inhibition and stimulation of β-glucosidase activity in Zn and Pb treatments, respectively). According to the GAMs, values of N_{min} increased after the second pollution event in Cd, Pb and Cu treatments (Figure 4.1c).

Regarding parameters of soil microbial biomass, we assessed the effect of repeated single-metal and multi-metal pollution events on bacterial and fungal gene abundance. Compared to control soil, Pb, Zn and multi-metal treatments significantly decreased bacterial gene abundance (Table 4.2); GAMs for values of bacterial gene abundance confirmed the marked inhibitory effect of Zn and multi-metal treatments (Figure 4.1d). On the other hand, all single-metal treatments led to lower values of fungal gene abundance (Table 4.2). As reflected by GAMs, in the first and second pollution event, the multi-metal treatment also decreased fungal gene abundance; in any case, this parameter increased after the third pollution event (Figure 4.1e). As far as the F:B ratio is concerned (the ratio of fungal to bacterial gene abundance), significantly higher values were observed under the multi-metal treatment, compared to control soil (Table 4.2). GAMs revealed that the F:B ratio drastically increased after the second pollution event for all single-metal treatments, to then decreased after the third pollution event (Figure 4.1f). In the multi-metal treatment, the F:B ratio increased throughout the experimental period (Figure 4.1f).

Table 4.2. Effect of treatments on soil microbial parameters at the end of the experiment (average value ± standard error; n = 3). Values followed by different letters are significantly different ($p<0.05$) according to Tukey-Kramer test. F:B: ratio of fungal to bacterial gene abundance; SQI: soil quality index.

	Acid phosphatase	β-glucosidase	Potentially mineralizable N	Bacterial gene abundance	Fungal gene abundance	F:B	SQI
	(mg ρ-nitrophenol kg^{-1} h^{-1})		(mg N-NH_4^+ kg^{-1} DW soil)	(× 10^8 copies g^{-1} DW soil)			
Control	1487 ± 33^{a}	333 ± 3^{ab}	38 ± 1^{a}	134.88 ± 7.79^{a}	1.64 ± 0.14^{a}	0.0125 ± 0.0016^{a}	94 ± 1^{a}
Cd	1478 ± 50^{a}	348 ± 3^{ac}	57 ± 3^{a}	100.49 ± 10.32^{ac}	0.85 ± 0.20^{bc}	0.0081 ± 0.0013^{a}	74 ± 6^{bc}
Pb	1595 ± 47^{a}	356 ± 4^{c}	49 ± 2^{a}	91.84 ± 8.10^{c}	0.50 ± 0.04^{c}	0.0056 ± 0.0008^{a}	67 ± 2^{bcd}
Cu	1165 ± 17^{b}	341 ± 1^{ac}	50 ± 2^{a}	128.25 ± 3.09^{ac}	0.74 ± 0.04^{bc}	0.0058 ± 0.0003^{a}	76 ± 1^{b}
Zn	948 ± 64^{b}	312 ± 4^{d}	47 ± 7^{a}	49.29 ± 6.07^{b}	0.35 ± 0.05^{c}	0.0077 ± 0.0016^{a}	51 ± 1^{d}
Multi-metal	656 ± 17^{c}	322 ± 3^{bd}	38 ± 1^{a}	24.93 ± 2.17^{b}	1.33 ± 0.12^{ab}	0.0532 ± 0.0018^{b}	57 ± 2^{cd}

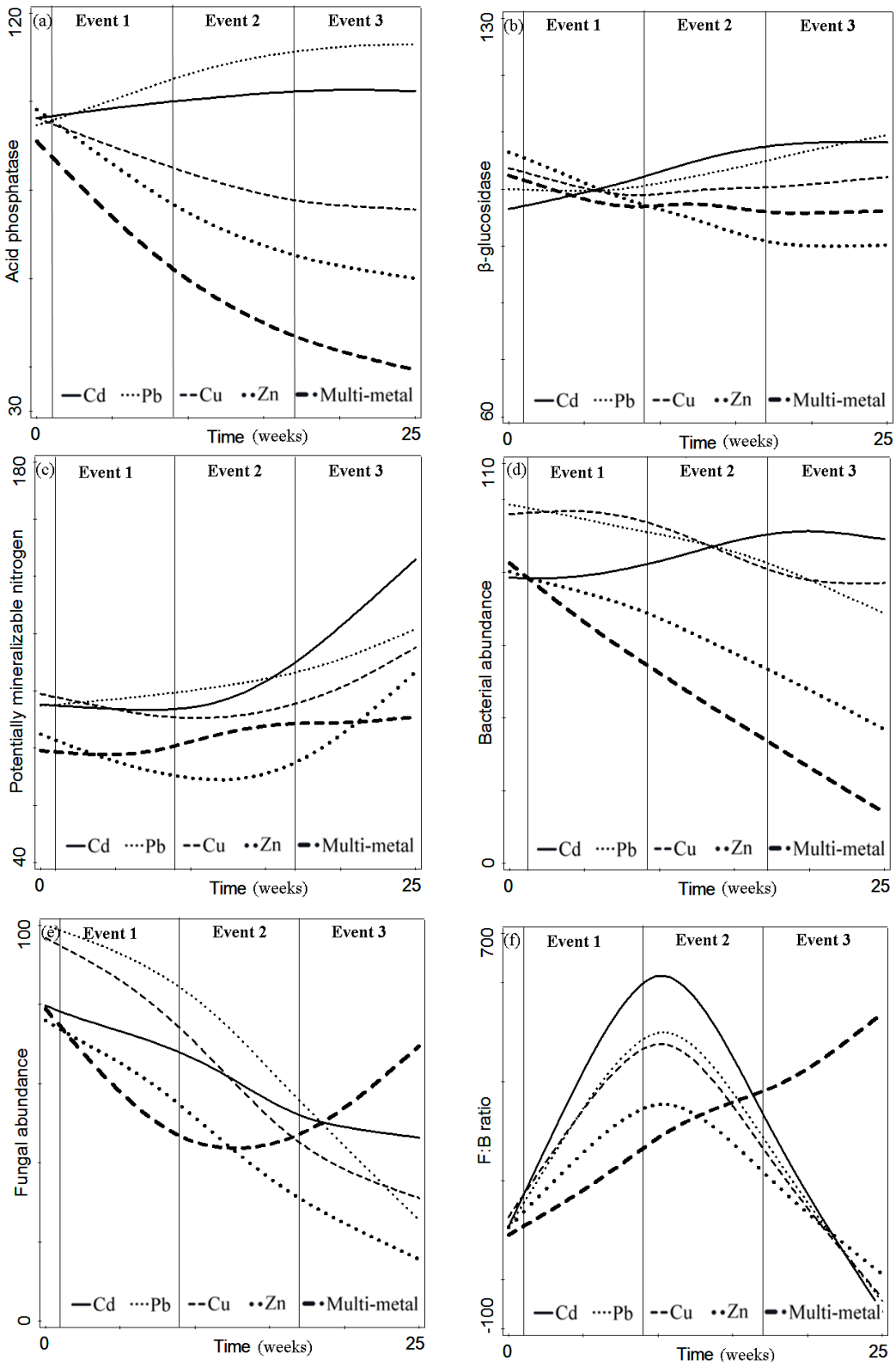


Figure 4.1. Generalized Additive Models for values of (a) acid phosphatase activity, (b) β-glucosidase activity, (c) potentially mineralizable N, (d) bacterial gene abundance, (e) fungal gene abundance, and (f) ratio of fungal to bacterial gene abundance, against time for each metal treatment. Relative values with respect to control treatment.

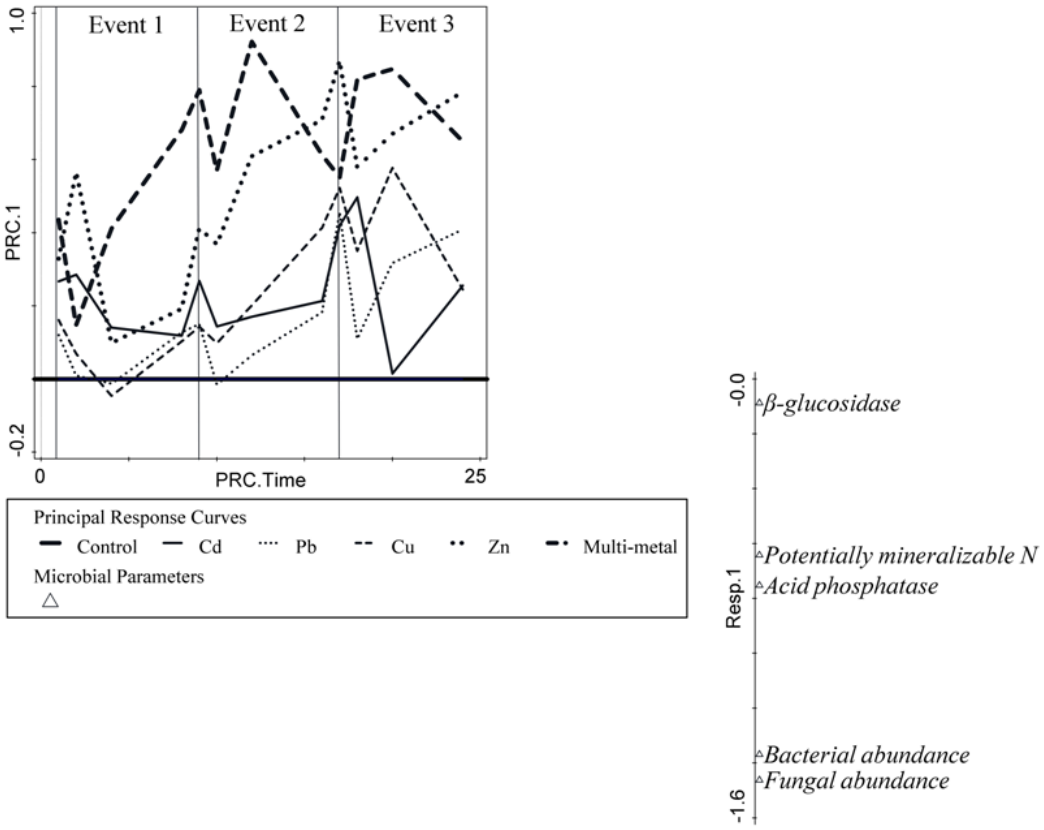


Figure 4.2. Principal Response Curves showing the effects of the different metal treatments on soil microbial parameters. The effects of factor "metal" are significant, according to Monte Carlo permutation test (F = 102, p = 0.001).

A Principal Response Curve (PRC) analysis was performed to assess the temporal response of the soil microbial communities to the metal treatments studied here (Figure 4.2). Measured microbial parameters were significantly related to the treatment effects captured by the PRC (p= 0.001). The PRC diagram shows small variations after the first pollution event under single-metal treatments (and larger variations under the multi-metal treatment). The second and third pollution events resulted in larger deviations from the control, particularly under Zn and multi-metal treatments. Besides, score values indicate which parameter has the greatest response to a given treatment. In our study, fungal and bacterial gene abundance showed the highest negative score, followed by acid phosphatase activity and N_{min}; thus, these parameters were most negatively affected, relative to controls, due to the pollution events. In contrast, β-glucosidase activity showed a small negative score, indicating that this parameter is likely to show a weak response or a response that is unrelated to that shown in Figure 4.2.

In order to provide a visual illustration of the overall effect of metal treatments on soil microbial parameters, a sun ray plot is presented in Figure 4.3. At the end of the experiment, values of acid phosphatase activity, bacterial and fungal gene abundance were in general lower under all metal treatments (single-metal and multi-metal). On the contrary, β-glucosidase activity hardly showed any variation as a consequence of metal treatments. Finally, values of N_{min} were in general higher in metal polluted soils, as compared to control soil.

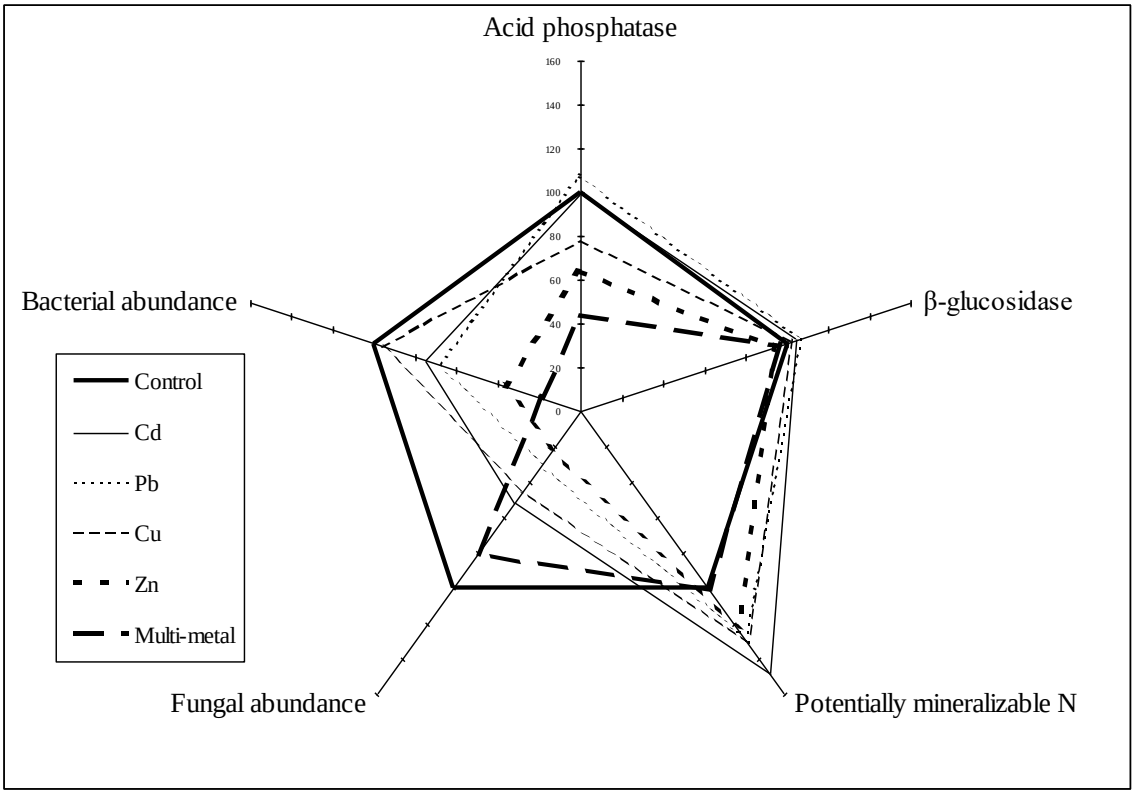


Figure 4.3. Sun ray plot of soil microbial parameters at the end of the experiment. A value of 100 corresponds to the mean value obtained for each specific parameter in the control treatment.

Finally, according to the Soil Quality Index (SQI), all metal treatments (single-metal and multi-metal) had a statistically significant adverse impact on soil quality (Table 4.2).

4.4 Discussion

4.4.1 Effect of repeated pollution events on metal bioavailability

$CaCl_2$-extractable metal concentrations in soil provide a good estimation of the fraction of metal available to biota (*i.e.*, metal bioavailability in soil) (Houba et al., 2000).

Although, as abovementioned, values of $CaCl_2$-extractable metal concentrations in both single-metal and multi-metal polluted soils increased progressively with successive pollution events (Table 4.1), no clear relationship between total metal concentrations and $CaCl_2$-extractable metal concentrations in soil was observed. It is a well-known fact that metal bioavailability in soil is strongly influenced by many different soil factors, such as pH, OM content, clay content, etc., as well as by metal sorption-desorption mechanisms (Lamb et al., 2009). Consequently, in order to better interpret the values of metal bioavailability in soil, it is important to take into consideration the sorption behaviour of the studied metals; in particular, the four metals included in our experiment can be classified according to their sorption behaviour as follows: Cu~Pb>Zn>Cd (Mertens et al., 2006). Copper has a high affinity for organic matter, with which it easily forms complexes due to the high stability constant of organic-Cu compounds; thus, only a small portion of the total Cu concentration in soil is present in the $CaCl_2$-extractable metal fraction (1.4-3.2% in our study). Lead exhibits a similar response to Cu in terms of relative bioavailability, owing to their similar sorption behaviour (1.4-2.0% in our study). On the contrary, the large proportion of Cd present in the $CaCl_2$-extractable metal fraction (32.5-37.9% in our study) reflects its higher bioavailability in soil, due to the fact that Cd does not seem to form strong complexes with organic matter. Similarly, Zhang et al. (2010b) reported that Zn is mainly found in weakly bound fractions (carbonate and reducible) in the soil, thus showing relatively high values of relative bioavailability. In our study, 7.1-20.4% of Zn appeared in the $CaCl_2$-extractable metal fraction.

In addition, in our experiment, values of relative metal bioavailability showed a slight tendency to increase progressively with successive pollution events. This increase was strong enough to hide the expected decline of metal bioavailability with ageing. Often, increasing residence leads to the development of inner sphere complexes, surface diffusion within micropores or surface precipitates (Aharoni and Sparks, 1991), resulting in a decrease in metal bioavailability. In our case, for Cu and Pb, the increase was substantially more pronounced in multi-metal *versus* single-metal treatments. As described above, Cu and Pb have a high affinity for soil organic matter and both can form strong specific bonds with electron-rich functional groups (Mbila et al., 2001), rendering them poorly bioavailable in soil; nonetheless, we observed that repeated multi-metal pollution events resulted in a dramatic increase in Cu and Pb relative

bioavailability values, which could be attributed to a flush of dissolved organic carbon (DOC) caused by the mortality of the soil microorganisms exposed to increasing levels of toxic metals. Dissolved organic carbon can decrease the sorption of Pb onto soil surfaces, either by competing for free metals and forming soluble organo-complexes or by being preferentially sorbed onto surfaces over competing metals (Giusquiani et al., 1998) . Other studies have demonstrated an increase in Cu bioavailability due to the formation of Cu complexes with OM-derived DOC (Brandt et al., 2010). Alternatively, when values of total metal concentration in soil increased as a result of repeated pollution events, a larger pool of pore-water metals, competing for surfaces and humic substances to bond with, was probably found in our metal polluted soils; therefore, a larger proportion of the total Cu and Pb concentration in soil remained in a $CaCl_2$-extractable form. All this concurs with the idea that values of total metal concentration are a poor indicator of metal mobility and bioavailability in soil (Barrutia et al., 2009; Lamb et al., 2009) and that to know the concentration of a given metal in its bioavailable form is crucial to understand its potential toxicity (Wang et al., 2007).

4.4.2 Effect of repeated pollution events on soil microbial parameters

In our study, we observed a relationship between metal treatments and the observed values of soil microbial parameters: indeed, in the variation partitioning analysis, metal treatment was the factor that explained most of the data variation (16% of the total variation). On the other hand, the SQI, calculated from the values of all the soil microbial parameters determined here (Table 4.2), can be interpreted as an integrative measurement of the combined effect of metals on both soil microbial biomass and activity, thereby providing valuable information on the overall microbial functionality of the metal-impacted soil. Similarly, the area and shape of the sun ray plot (Figure 4.3) provide an integrated fingerprinting to assess the adverse impact of the repeated pollution events on the overall soil microbial functionality. In our study, according to the SQI, all metal treatments had a statistically significant adverse impact on soil quality, as reflected by the values of soil microbial biomass and activity parameters. In any case, although the SQI is indeed very useful to integrate different results, it must be interpreted with caution as it is only a simplified reflection of the extremely complex soil ecosystem and, inevitably, entails a loss of useful information about the response of each specific parameter considered during the calculation of the SQI.

According to the PRC analysis (Figure 4.2), which summarizes all the measured variation in soil microbial parameters throughout the 26-week experiment, we can conclude that the repeated pollution events with the Zn and multi-metal treatments had a substantial negative effect on the soil microbial communities. Similarly, Epelde et al. (2009b) found that 1,000 mg Zn kg^{-1} DW, and especially 2,000 and 4,000 mg Zn kg^{-1} DW, clearly affected the capacity of the cultivable portion of the soil heterotrophic microbial community to utilize carbon substrates, according to Biolog Ecoplates™ data. On the contrary, despite its well-known toxicity to living organisms (Dong et al., 2007), in our study, Cd did not cause a clear negative impact on the measured parameters, with the exception of fungal gene abundance (Table 4.2).

The observed effects of the repeated metal pollution events highlight the differences in sensitivity and response speed of each soil microbial parameter in the presence of toxic metals. Thus, an immediate negative effect was observed for acid phosphatase activity under Pb, Zn and multi-metal treatments (*i.e.*, negative effects were already observed after the first pollution event) (Figure 4.1). Soil enzymes have been recommended as standard biochemical indicators to assess the quality of metal-polluted soils (Hinojosa et al., 2008). As aforesaid, extractable metal forms are considered to be responsible for the metal-induced inhibitory effects on soil microbial parameters, including soil enzyme activities (Rusk et al., 2004). Then, surprisingly, Cd, a highly bioavailable metal, did not cause a negative impact on acid phosphatase activity (Table 4.2), showing that the same enzyme activity may respond differently to different metals and that the responses of different enzymes to the same metal can also vary (Zhang et al., 2010a). In this sense, β-glucosidase activity did not show significant changes to metal treatments throughout the experiment (Figure 4.1), unlike acid phosphatase activity.

On the other hand, values of β-glucosidase activity showed a slight increase throughout the experiment (Figure 4.1) under Pb and Cd treatments, in accordance with Tica et al. (2011), who found a positive correlation between β-glucosidase activity and metal bioavailable concentrations. β-glucosidase has a crucial role in the soil carbon cycle: it catalyses the hydrolysis of various β-glucosides during the decomposition of organic materials (Schinner et al., 1996), producing hydrolysis products which are then important sources of energy for soil microorganisms; in this respect, this enzyme activity has a most important role in polluted soils, where microorganisms have a higher carbon requirement for repair and maintenance (Lamb et al., 2009). Indeed, an increase

in the bioavailable fraction of metals leads to a deterioration of the environmental conditions for the exposed soil microorganisms and, concomitantly, increases their carbon requirement. In any event, as previously reported (Lamb et al., 2009; Zhang et al., 2010a), our data show that metal exposure can either increase or decrease the values of soil enzyme activities, depending on the specific enzyme activity, metal and soil properties.

According to GAMs, values of potentially mineralizable N appear to increase progressively with successive pollution events (Figure 4.1). As a consequence of the successive pollution events, the increasing values of total and bioavailable metal concentrations found in soil most likely caused higher levels of metal-induced toxicity and microbial cell death; concomitantly, the flush of organic N released from dead microbial cells might be responsible for the observed higher values of N_{min} (Kao et al., 2006). In any case, at the end of the experiment, no significant differences in N_{min} values were found under single-metal or multi-metal treatments, as compared to control soil (Table 4.2), indicating a low impact of metal treatments on N mineralization, in disagreement with data from Kao et al. (2006). Then, our results support the idea of N mineralization as a process relatively insensitive to disturbances, due to the fact that a wide variety of microorganisms are involved in such process (Winding et al., 2005).

The adverse impact of toxic metals on soil microbial activity can be caused directly through, for instance, inactivation of extracellular enzymes, or indirectly through their effect on soil microbial populations (Lee et al., 2009). In our study, microbial biomass parameters were most severely affected by the repeated metal pollution events, reflecting the adverse impact of metals in the development and survival of microorganisms in metal polluted soils (Smolders et al., 2003). In this respect, we observed a high sensitivity of fungal and bacterial gene abundance to repeated metal pollution events, as reflected by the results of our PRC analysis (these two parameters, followed by acid phosphatase activity, were most affected by metal treatments).

4.5 Conclusions

Both single-metal and multi-metal repeated pollution events had a significant impact on soil quality (as reflected by the Soil Quality Index calculated from the values of all soil microbial biomass and activity parameters). Parameters of microbial biomass, together with acid phosphatase activity, showed a high sensitivity and quick response to repeated

metal treatments. On the other hand, values of relative bioavailability ($[metal]_{bio}/[metal]_{tot}$) increased with successive single-metal and multi-metal pollution events. It was concluded that the application of repeated single-metal *versus* multi-metal contamination events is very useful to monitor both the response of soil microbial communities to metal pollution and to better understand the behaviour and toxic effects of each metal on microbially-driven soil processes.

05 | ECOSYSTEM SERVICES AND PLANT PHYSIOLOGICAL STATUS DURING ENDOPHYTE-ASSISTED PHYTOREMEDIATION OF METAL CONTAMINATED SOIL

5. ECOSYSTEM SERVICES AND PLANT PHYSIOLOGICAL STATUS DURING ENDOPHYTE-ASSISTED PHYTOREMEDIATION OF METAL CONTAMINATED SOIL

Burges A, Epelde L, Blanco F, Becerril JM, Garbisu C. Ecosystem services and plant physiological status during endophyte-assisted phytoremediation of metal contaminated soil. Aceppted in ***Science of the Total Environment****.* DOI: 10.1016/j.scitotenv.2016.12.146.

Abstract

Mining sites shelter a characteristic biodiversity with large potential for the phytoremediation of metal contaminated soils. Endophytic plant growth-promoting bacteria were isolated from two metal-(hyper)accumulator plant species growing in a metal contaminated mine soil. After characterizing their plant growth-promoting traits, consortia of putative endophytes were used to carry out an endophyte-assisted phytoextraction experiment using *Noccaea caerulescens* and *Rumex acetosa* (singly and in combination) under controlled conditions. We evaluated the influence of metal phytoextraction on soil physicochemical and microbial properties, as well as plant physiological parameters and metal concentrations. Data interpretation through the grouping of soil properties within a set of ecosystem services was also carried out. When grown together, we observed a 41 and 16% increase in the growth of *N. caerulescens* and *R. acetosa* plants, respectively, as well as higher values of metal phytoextraction (particularly, Zn) and soil microbial biomass and functional diversity. Inoculation of the consortia of putative endophytes did not lead to higher values of plant metal uptake, but it improved the plants´ physiological status, by increasing the content of chlorophylls and carotenoids by up to 28 and 36%, respectively, and stimulated soil microbial communities (higher values of acid phosphatase activity, bacterial and fungal abundance, and structural diversity). The positive effects of plant growth and endophyte inoculation on soil properties were reflected in an enhancement of some ecosystem services.

5.1 Introduction

Phytoextraction, *i.e.* the use of plants to extract heavy metals from soil, is a promising phytoremediation option for metal contaminated sites, among other reasons, owing to its low cost and environmentally-friendly character (Chen et al., 2010; Epelde et al.,

2012b). The amount of metal phytoextracted depends on (i) its concentration in the harvestable parts of plants and (ii) plant biomass (Barrutia et al., 2009). However, the small biomass and slow growth of many (hyper)accumulators, as well as a low soil metal bioavailability, can limit the effectiveness of phytoextraction (Rajkumar et al., 2009). This has brought up the necessity to explore possibilities for stimulating plant growth and metal uptake during phytoextraction.

Mining sites shelter a characteristic biodiversity, adapted to the harsh conditions commonly present in these areas, with large potential for the phytoremediation and phytomanagement of metal contaminated soils (Barrutia et al., 2011). Thus, the selection of the right (pseudo)metallophytes from mining areas (these species are characterized by their metal tolerance and high capacity to accumulate heavy metals in their tissues) is crucial for effective phytoextraction (Barrutia et al., 2009). On the other hand, it has been widely reported (Mastretta et al., 2009; Chen et al., 2010; Luo et al., 2011; Xinxian et al., 2011; Zhang et al., 2011) that the application of plant growth-promoting rhizobacteria and bacterial endophytes has large potential for the phytoremediation and phytomanagement of metal contaminated sites, owing to their ability to increase plant biomass as well as metal uptake and tolerance.

The main objective of soil metal remediation is not only to remove the metals from the soil or to render them harmless but also to restore soil functioning (Epelde et al., 2014b). Soil microbial properties have been reported as suitable biological indicators of the effectiveness of metal phytoremediation (Epelde et al., 2008a). Besides, it has been proposed (Velasquez et al., 2007; Rutgers et al., 2012) to assess soil quality according to the concept of ecosystem services. In this respect, we proposed to group soil microbial parameters within a set of ecosystem services in order to facilitate interpretation and to provide stability to phytoremediation monitoring programs (Garbisu et al., 2011; Epelde et al., 2014a).

The aim of this study was to assess the effectiveness of endophyte-assisted phytoextraction of metal contaminated soil, with particular emphasis on the recovery of ecosystem services. The following steps were undertaken: (i) isolation of bacterial putative endophytes from two native plant species (*Noccaea caerulescens:* hyperaccumulator; *Rumex acetosa:* accumulator) collected from an abandoned Pb/Zn mine; (ii) identification of suitable consortia of bacterial putative endophyte strains with plant growth-promoting traits and; (iii) investigation of endophyte-assisted

phytoextraction of Pb/Zn/Cd under growth chamber conditions using *N. caerulescens* and *R. acetosa*, singly and in combination.

5.2 Materials and methods

5.2.1 Site description and plant sampling

The study started in an abandoned Pb/Zn mine (Coto "Txomin", province of Biscay, Spain, 43º43´N, 3º26´W) that presents very high levels of toxic heavy metals (*i.e.*, Cd, Pb and Zn) in the soil. For a more detailed description and characterization of the mine, see Barrutia et al. (2011). Healthy plants of *Noccaea caerulescens* J. & C. Presl. and *Rumex acetosa* L. were randomly collected from the mine. Plant samples were thoroughly washed, as described in Burges et al. (2016).

5.2.2 Isolation, characterization and identification of endophytic bacteria

Bacterial putative endophytes were isolated from roots, stems and leaves, according to Surette et al. (2003). Plant tissues were surface disinfected by immersion in bleach (5% available chlorine) for 3 min, 3% hydrogen peroxide solution for 3 min, and finally rinsed three times with sterile distilled water. Roots, stems and leaves (0.5 g FW) were then homogenized in 10 ml 3x Ringer's solution (Surette et al., 2003), using an ethanol-sterilized mortar and pestle and adding sterile quartz sand to improve wall disruption, and incubated at room temperature in an orbital shaker for 1 h. Tissue extracts were serially diluted to 10^{-3} with 3x Ringer's solution. Aliquots of 100 µl of each dilution were plated out in duplicate onto tryptic soy agar (TSA) and Luria Bertani's (LB) agar plates supplemented with 100 mg l-1 cycloheximide. Plates were then incubated at 28 ºC for 72 h. Based on colony morphotype (colour, size and shape), we isolated 31 putative endophytic bacterial strains. Culture stocks at -80 ºC were prepared following Burges et al. (2006).

All endophytic bacterial strains were tested for their 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (Penrose and Glick, 2003). ACC deaminase activity has been suggested to be, arguably, a major mechanism that bacterial endophytes use to promote plant growth, because it might ameliorate plant stress by efficiently blocking ethylene production (Hardoim et al., 2008; Glick, 2014). In consequence, only those endophytic bacterial strains containing ACC deaminase activity were tested for the other plant growth-promoting traits studied here: indole-3-acetic acid (IAA) production (Becerra-Castro et al., 2011), siderophore production

(Schwyn and Neilands, 1987) and phosphate solubilising activity (Nautiyal, 1999). For the determination of indole-3-acetic acid production, putative endophytes were incubated in liquid medium supplemented with 0.5 mg ml^{-1} L-tryptophan for 5 days, and subsequently quantified spectrophotometrically after incubating the cell suspension's supernatant with Salkowski's reagent for 25 min. Metal and salt tolerance were determined according to Long et al. (2011) and Rashid et al. (2012), respectively, as described in Burges et al. (2016), and a phenotypic characterization was carried out using GEN III MicroPlates™ (Biolog Inc., Hayward, USA)]. Taxonomic identification was determined as detailed in Burges et al. (2016).

5.2.3 Soil characterization and experimental design

The experiment was carried out in pots containing heavy metal contaminated soil from the abandoned mine. Soil was collected from the top layer (0-20 cm) and immediately transported to our laboratory where visible roots were removed. The mine soil was sieved to <4 mm and thoroughly mixed using a cement mixer. Soil sub-samples were air-dried and sieved to <2 mm prior to physicochemical characterization (MAPA, 1994). The soil was characterized as a sandy-loam with the following properties: pH = 6.8; OM (%) = 4.9; total N (%) = 0.21; extractable P (mg kg^{-1} dry weight-DW soil) = 2.2; and extractable K^+ (mg kg^{-1} DW soil) = 54. Total concentrations of metals (Cd, Pb and Zn) were determined using an Absorption Atomic Spectrometer (Spectra AA-250 plus, Varian, Australia) following aqua regia digestion (McGrath and Cunliffe, 1985). Total concentrations of heavy metals in soil were (mg kg^{-1} DW soil): 12.9, 6,345 and 18,284 for Cd, Pb and Zn, respectively.

Two species of metallophytes were used: (i) the hyperaccumulator *N. caerulescens* and (ii) the accumulator *R. acetosa*. Seeds of *N. caerulescens* (Hernandez-Allica et al., 2006) and *R. acetosa* (Barrutia et al., 2009) were collected from the abandoned mine and germinated for 1 month on a mixture of perlite and vermiculite (1:3; v/v, moistened with deionized H_2O) under the following growth chamber conditions: 14/10 h light/dark cycle, 20/16 ºC day/night temperature, 70% relative humidity, and a photosynthetic photon flux density of 150 µmol photon m^{-2} s^{-1}. Subsequently, plants were transplanted to a mixture of perlite and vermiculite (2:3; v/v), moistened with a nutrient solution [1 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM K_2HPO_4, 0.1 mM KCl, 2 mM MES-HCl buffer pH 6, 1m M KOH, 10 µM H_3BO_3, 0.2 µM $Na_2MoO_4 \cdot 2H_2O$, 1.8 µM $MnSO_4 \cdot 4H_2O$, 0.3 µM $CuSO_4 \cdot 5H_2O$, 0.5 µM

$NiSO_4 \cdot 6H_2O$, 100 µM Fe-EDDHA] supplemented with 1 µM $ZnSO_4 \cdot 7H_2O$ or 5 µM $ZnSO_4 \cdot 7H_2O$ for *N. caerulescens*, and allowed to grow for 1 month. Equally developed plants were selected for the experiment.

Endophytic bacterial strains that showed the best performance for any of the plant growth-promoting traits described above were used to form the consortia for inoculation (Table 5.1): isolates NR1, NL1 and NL2 from *N. caerulescens* for inoculation of *N. caerulescens*; and isolates RR1, RR3 and RL2 from *R. acetosa* for inoculation of *R. acetosa*. The consortia of putative endophytes were obtained by growing the isolates separately, as described in Burges et al. (2016), and mixing equal volumes of individual bacterial cultures. For inoculation, *N. caerulescens* and *R. acetosa* plants were soaked for 2 h in the corresponding bacterial consortium following Chen et al. (2010). Four plants were transferred to pots containing 1.4 kg (DW) soil. Control (non-inoculated) plants were soaked in sterile 0.85% KCl.

A total of 7 treatments were conducted in quadruplicate: (1) N-E: four plants of *N. caerulescens* inoculated with the putative endophyte consortium; (2) N-N: four plants of *N. caerulescens*, without endophyte inoculation; (3) R-E: four plants of *R. acetosa* inoculated with the putative endophyte consortium; (4) R-N: four plants of *R. acetosa*, without endophyte inoculation; (5) NR-E: two plants of *N. caerulescens* + two plants of *R. acetosa*, inoculated with both endophyte consortia combined together; (6) NR-N: two plants of *N. caerulescens* + two plants of *R. acetosa*, without endophyte inoculation; (7) UP: unplanted. Rhizon samplers (Rhizosphere Research Products, The Netherlands) were inserted in the soil at a 15 cm soil depth for sampling of pore water. Plants were then allowed to grow in the growth chamber, as described in Burges et al. (2016).

5.2.4 Plant parameters

After 24 weeks of growth, photochemical efficiency (Fv/Fm) was measured, and photoprotective pigments [carotenoids (Car), neoxanthin (Neo), violaxanthin (Vio), antheraxanthin (Ant), zeaxanthin (Zea) and lutein (Lut)], photosynthetic pigments [chlorophylls a and b (Chl)] and lipophilic antioxidants [α-tocopherol (α-Toc)] were determined following García-Plazaola and Becerril (2001).

Shoots and roots were separately harvested and dry weights (DW) were calculated according to Burges et al. (2016). Heavy metal concentrations (Cd, Pb and Zn) in plant tissues were determined following Zhao et al. (1994).

5.2.5 Soil physicochemical properties

Pore water samples were collected at the end of the experiment using Rhizon samplers for the determination of soluble heavy metal concentrations (Cd, Pb and Zn) using an Absorption Atomic Spectrometer.

Soil was collected, sieved and processed as explained in Burges et al. (2016). Soil water content at field capacity was measured according to Richards (1948). Total and $CaCl_2$-extractable (0.01 M) concentrations of heavy metals (Cd, Pb and Zn) in soil were determined according to McGrath and Cunliffe (1985) and Houba et al. (2000), respectively. Total carbon (C) and total nitrogen (N) contents were measured following ISO 10694 (1995) and ISO 13878 (1998), respectively.

5.2.6 Soil microbial properties

Soils were sieved to <2 mm and stored at 4 ºC (Burges et al., 2016). β-glucosidase, arylsulphatase and acid phosphatase enzyme activities were determined following Dick (1997) and Taylor et al. (2002); β-glucosaminidase activity was determined following Parham and Deng (2000); urease activity was measured according to Kandeler and Gerber (1988); dehydrogenase activity was measured following ISO 23753-2 (2005); fluorescein diacetate (FDA) hydrolytic activity was estimated following Shaw et al. (2006); amidase and arginine deaminase were determined as described by Acosta-Martínez and Tabatabai (2000) and Kandeler (1996), respectively; and protease activity was quantified according to Geisseler and Horwath (2008). Microbial biomass carbon (MBC) was determined following Vance et al. (1987). Basal and substrate-induced respiration (SIR) were measured according to ISO 16072 (2002) and ISO 17155 (2002), respectively, as modified by Epelde et al. (2008a). Soil suppressiveness, using *Rhizoctonia solanum* as reference organism, was determined following Grünwald et al. (1997) as described in Nuñez-Zofío et al. (2012). Community-level physiological profiles (CLPPs) were determined using Biolog EcoPlates™ (Biolog Inc., USA) following Epelde et al. (2008a). Number of utilized substrates (NUS), average well colour development (AWCD), and Shannon's diversity (H') and Pielou's evenness (J') indexes were calculated after 50 h of incubation (*i.e.*, the time of maximal growth in the Biolog EcoPlates™).

For molecular analysis, DNA was extracted following Epelde et al. (2016). Estimations of the abundance of 16S rDNA gene fragments for total bacteria (primers

Ba519F and Ba907R; Lueders et al., 2004a; 2004b) and 18S rDNA gene fragments for total fungi (primers Fung5F and FF390R; Lueders et al., 2004a; 2004b) were determined using real-time PCR measurements according to Epelde et al. (2014b). Community-level genetic profiles were obtained with ARISA (automated method of ribosomal intergenic spacer analysis) following Cardinale et al. (2004) for bacteria and Ranjard et al. (2000) for fungi, as indicated in Welkie et al. (2010).

5.2.7 Statistical analysis

The effects of experimental factors ("plant species": *N. caerulescens*, *R. acetosa* or both species; "endophyte inoculation": absence or presence of endophyte inoculation) and their interactions on plant parameters, soil physicochemical properties and soil microbial properties were evaluated by two-way ANOVA using IBM SPSS Statistics 19.0. When a significant effect or interaction was observed, differences (P<0.05) among factors or levels of factors were tested using the Fisher's Least Significant Difference (LSD) test. In order to explore relationships between treatments and soil quality, data on soil microbial properties were analysed by redundancy analysis (RDA) and variation partitioning analysis with Canoco 5 (ter Braak and Šmilauer, 2012). Similarly, RDA and variation partitioning were used to investigate the influence of treatments on plant physiological parameters.

The provision of ecosystem services was evaluated from data on soil physicochemical and microbial properties, as described in Burges et al. (2016), according to the formula proposed by Epelde et al. (2014b):

$$ES = 10^{\log m + \frac{\sum_{i=1}^{n} |\log n_i - \log m|}{n}}$$

where m is the control value (set to 100%) and n corresponds to the measured values for each parameter as percentage of the control value. Soil physicochemical and microbial properties were grouped within ecosystem services as follows: (1) *soil biodiversity*: richness (S), Shannon's diversity (H') and Pielou's evenness (J') indexes of bacterial and fungal communities obtained from ARISA profiles; and AWCD, NUS, H' and J' indexes from Biolog EcoPlates™; (2) *nutrient cycling*: basal respiration and enzyme activities; (3) *carbon storage*: total C content, bacterial and fungal gene abundance, and

MBC; (4) *water flow regulation*: water content at field capacity*;* (5) *water purification*: soluble and $CaCl_2$-extractable metal concentrations; (6) *contamination control*: $CaCl_2$-extractable and total heavy metal concentrations; (7) *pest control*: soil suppressiveness; (8) *fertility maintenance*: total N and organic C content. The arithmetic mean of these 8 ecosystem services was calculated as an index of soil quality (SQI). Significant differences among different treatments for the abovementioned ecosystem services were studied by two-way ANOVA.

5.3 Results

5.3.1 Isolation of bacterial putative endophytes

From the 31 endophytic bacterial strains isolated from the interior tissues of *N. caerulescens* and *R. acetosa*, a total of 12 isolates contained ACC deaminase activity ranging from 0.51 to 3.28 µmol mg^{-1} h^{-1}. These 12 isolates were (i) analysed in terms of plant growth-promoting traits and (ii) then phenotypically characterized (Table 5.1). According to their plant growth-promoting traits, 3 isolates from each metallophyte species were chosen for inoculation: NR1, NL1 and NL2 (*Variovorax* sp., *Micrococcus* sp. and *Microbacterium* sp., respectively) from *N. caerulescens*; RR1, RR3 and RL2 (Pseudomonas sp., Microbacterium sp. and Microbacterium sp., respectively) from R. acetosa (Table 5.1).

5.3.2 Effects on plant parameters

In order to assess the effects and interactions of the different experimental factors ("plant species": *N. caerulescens*, *R. acetosa* or both species; "endophyte inoculation": absence or presence of endophyte inoculation) on plant parameters, a two-way ANOVA was conducted. According to this ANOVA, the "plant species" factor had a significant effect on total plant biomass for both *N. caerulescens* and *R. acetosa* (Figure 5.1). Accordingly, Fisher's LSD-test was used to establish the significance of the differences among means: total plant biomass of *N. caerulescens* and *R. acetosa* were significantly higher in NR pots, particularly due to the higher growth of aerial parts (*i.e.*, shoot biomass). No effect of endophyte inoculation was observed on plant biomass of either *N. caerulescens* or *R. acetosa*.

Table 5.1. Selection of bacterial putative endophytes: plant growth-promoting traits, phenotypic characterization and identification. In bold, best performance values used to select the members of the consortia

			Plant growth-promoting traits				Phenotypic characterization							
Isolate	**Host**	**Organ**	**ACC**	**IAA**	**Sid**	**PO_4**	**Salt**	**Cd**	**Pb**	**Zn**	**NUCS**	**NARS**	**H'**	**Identification**
NR1	*N. caerulescens*	Root	**3.28 ± 0.40**	13.5 ± 0.1	-	-	0.5	5	6	5	24	1	4	*Variovorax* sp.
NR2	*N. caerulescens*	Root	0.51 ± 0.06	nd	-	-	2	2	4	10	12	2	4	*Microbacterium* sp.
NS3	*N. caerulescens*	Stem	1.31 ± 0.10	nd	-	-	2	1	4	5	13	2	4	*Microbacterium* sp.
NS9	*N. caerulescens*	Stem	0.41 ± 0.07	nd	-	-	7	0.5	4	1	22	0	2	*Brevibacterium* sp.
NL1	*N. caerulescens*	Leaf	0.74 ± 0.23	**58.9 ± 1.3**	-	-	5	1	4	2.5	1	1	0	*Micrococcus* sp.
NL2	*N. caerulescens*	Leaf	0.48 ± 0.08	13.8 ± 0.1	-	+	3	0.5	6	5	2	2	1	*Microbacterium* sp.
NL4	*N. caerulescens*	Leaf	0.67 ± 0.03	nd	-	-	7	0.5	6	0.5	17	0	3	*Brevibacterium* sp.
RR1	*R. acetosa*	Root	0.63 ± 0.10	30.8 ± 0.1	+	+	4	2	8	5	1	5	0	*Pseudomonas* sp.
RR2	*R. acetosa*	Root	0.66 ± 0.09	33.0 ± 0.1	+	+	4	2	6	5	1	5	0	*Pseudomonas* sp.
RR3	*R. acetosa*	Root	**1.04 ± 0.03**	35.1 ± 0.5	-	-	3	0.5	2	5	42	2	5	*Microbacterium* sp.
RS1	*R. acetosa*	Stem	1.01 ± 0.21	83.5 ± 8.0	-	+	2	1	6	5	29	2	5	*Plantibacter* sp.
RL2	*R. acetosa*	Leaf	0.89 ± 0.11	**84.7 ± 3.7**	-	-	2	1	6	5	25	2	5	*Microbacterium* sp.

ACC, 1-aminocyclopropane-1-carboxylate deaminase activity (µmol mg^{-1} h^{-1}); IAA, indole-3-acetic acid production (µ$g^{-1}ml^{-1}$); Sid, siderophore production; PO_4, phosphate solubilising activity; Salt, salt tolerance (%); Cd, Maximal Tolerable Concentration (MTC) (mM Cd); Pb, MTC (mM Pb); Zn, MTC (mM Zn); NUCS, number of utilised carbon substrates; NARS, number of antibiotic resistance substrates; H', Shannon´s index; nd, not detectable

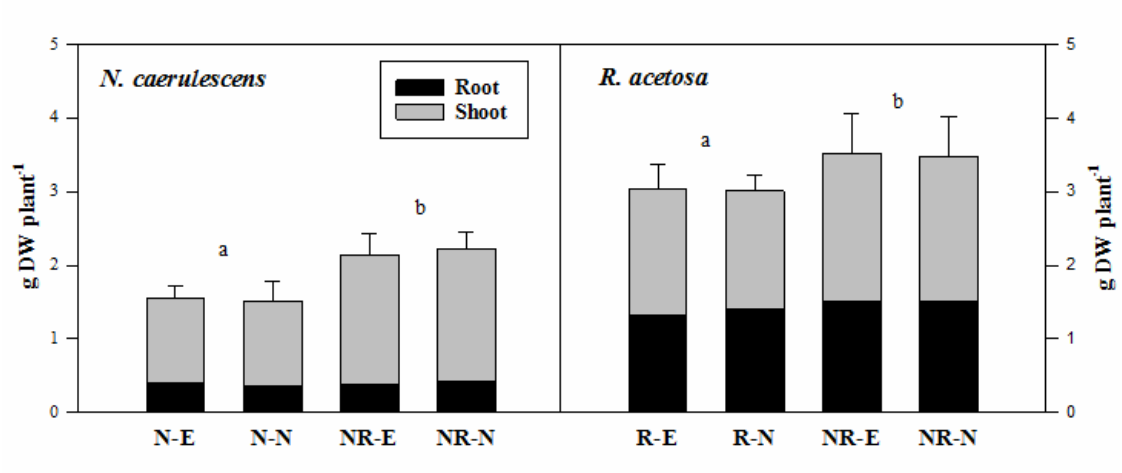


Figure 5.1. Effect of treatments on shoot and root biomass (g DW plant^{-1}) of *N. caerulescens* and *R. acetosa*. Values of total plant biomass are represented by the total bar area in each treatment (n=4) ± SD. Treatments with different letters are significantly (P<0.05) different according to Fisher's LSD-test. Probability values from two-way ANOVA (ns: non-significant; *, ** and *** represent significances of P<0.05, 0.01 and 0.001, respectively) for shoot, root and total biomass of *N. caerulescens*: plant, ***, ns, ***; endophyte, ns, ns, ns; plant x endophyte, ns, ns, ns; and of *R. acetosa*: plant, *, ns, *; endophyte, ns, ns, ns; plant x endophyte, ns, ns, ns. N-E: four plants of *N. caerulescens* inoculated with the endophyte consortium; N-N: four plants of *N. caerulescens*, without endophyte inoculation; R-E: four plants of *R. acetosa* inoculated with the endophyte consortium; R-N: four plants of *R. acetosa*, without endophyte inoculation; NR-E: two plants of *N. caerulescens* + two plants of *R. acetosa*, inoculated with both endophyte consortia combined together; NR-N: two plants of *N. caerulescens* + two plants of *R. acetosa*, without endophyte inoculation; UP: unplanted.

The effects of the experimental factors on plant physiological parameters are shown in Table 5.2. According to the variation partitioning and RDA analyses (Supplementary Figure 5.1a; F = 9.2, P<0.01), the "plant species" factor accounted for 24% of the explained variation in *N. caerulescens*; in NR pots, *N. caerulescens* plants showed higher values of photochemical efficiency (Fv/Fm), chlorophyll content (Chl) and some photoprotective pigments (*i.e.*, Neo, Lut, Car). On the contrary (Supplementary Figure 5.1b; F = 8.2, P<0.01), in NR pots, *R. acetosa* plants showed lower values of Chl and photoprotective pigments (but higher values of α-Toc); in this case, the "plant species" factor explained 39% of the variation.

Putative endophyte inoculation increased the content of photoprotective pigments and Chl. This positive effect was also reflected in the lower values of α-Toc content, particularly in NR pots, as indicated by the significant interaction found between "plant species" and "endophyte inoculation" for this parameter. According to the variation partitioning analysis, endophyte inoculation explained 35 and 17% of the variation in physiological parameters of *N. caerulescens* and *R. acetosa*, respectively.

Table 5.2. Effect of treatments on plant physiological parameters (Chl, VAZ, A+Z/VAZ, Car and α-Toc in nmol g^{-1} FW). Mean values (n = 4) ± SD. Probability values from two-way ANOVA (ns: non-significant) for the effects of "plant species" and "endophyte inoculation" and their interactions are shown below. N-E: four plants of *N. caerulescens* inoculated with the endophyte consortium; N-N: four plants of *N. caerulescens*, without endophyte inoculation; R-E: four plants of *R. acetosa* inoculated with the endophyte consortium; R-N: four plants of *R. acetosa*, without endophyte inoculation; NR-E: two plants of *N. caerulescens* + two plants of *R. acetosa*, inoculated with both endophyte consortia combined together; NR-N: two plants of *N. caerulescens* + two plants of *R. acetosa*, without endophyte inoculation

	N. caerulescens						***R. acetosa***					
	Fv/Fm	**Chl**	**VAZ**	**A+Z/VAZ**	**Car**	**α-Toc**	**Fv/Fm**	**Chl**	**VAZ**	**A+Z/VAZ**	**Car**	**α-Toc**
N-E	0.45 ± 0.08	709 ± 51	55 ± 6	0.14 ± 0.04	299 ± 32	182 ± 28						
N-N	0.57 ± 0.03	499 ± 114	38 ± 8	0.20 ± 0.03	187 ± 17	201 ± 24						
R-E							0.67 ± 0.06	1373 ± 194	72 ± 5	0.07 ± 0.01	387 ± 49	110 ± 25
R-N							0.75 ± 0.01	1197 ± 163	64 ± 11	0.07 ± 0.01	349 ± 37	106 ± 3
NR-E	0.67 ± 0.06	878 ± 98	60 ± 9	0.16 ± 0.02	342 ± 52	142 ± 14	0.72 ± 0.04	1184 ± 141	63 ± 6	0.06 ± 0.00	340 ± 37	149 ± 26
NR-N	0.70 ± 0.06	753 ± 125	47 ± 7	0.08 ± 0.01	286 ± 42	224 ± 23	0.74 ± 0.04	797 ± 64	47 ± 5	0.11 ± 0.02	240 ± 20	195 ± 5
Plant (P)	<0.001	<0.01	ns	<0.01	<0.01	ns	ns	<0.01	<0.01	<0.05	<0.01	<0.001
Endophyte (E)	<0.05	<0.01	<0.01	ns	<0.01	<0.01	<0.05	<0.01	<0.01	<0.01	<0.01	<0.01
P x E	ns	ns	ns	<0.001	ns	<0.01	ns	ns	ns	<0.01	ns	<0.01

Fv/Fm: photochemical efficiency; Chl: chlorophylls a and b; V: violaxanthin; A: antheraxanthin; Z: zeaxanthin; Car: carotenoids; α-Toc: α-tocopherol.

Table 5.3. Effect of treatments on shoot metal concentration (mg kg^{-1} DW), translocation factor, bioaccumulation factor and bioconcentration factor of *N. caerulescens* and *R. acetosa*. Mean values (n = 4) ± SD. Probability values from two-way ANOVA (ns: non-significant) for the effects of "plant species" and "endophyte inoculation" and their interactions are shown below. N-E: four plants of *N. caerulescens* inoculated with the endophyte consortium; N-N: four plants of *N. caerulescens*, without endophyte inoculation; R-E: four plants of *R. acetosa* inoculated with the endophyte consortium; R-N: four plants of *R. acetosa*, without endophyte inoculation; NR-E: two plants of *N. caerulescens* + two plants of *R. acetosa*, inoculated with both endophyte consortia combined together; NR-N: two plants of *N. caerulescens* + two plants of *R. acetosa*, without endophyte inoculation.

	Shoot Metal Concentration			**Translocation Factor**			**Bioaccumulation Factor**			**Bioconcentration Factor**		
	Cd	**Pb**	**Zn**	**Cd**	**Pb**	**Zn**	**Cd**	**Pb**	**Zn**	**Cd**	**Pb**	**Zn**
N. caerulescens												
N-E	16.9 ± 3.2	373 ± 29	12475 ± 1675	1.29 ± 0.23	0.19 ± 0.05	3.47 ± 0.93	1.50 ± 0.16	0.10 ± 0.02	0.88 ± 0.15	1.17 ± 0.15	0.55 ± 0.08	0.27 ± 0.04
N-N	18.0 ± 3.7	430 ± 93	10676 ± 1993	1.43 ± 0.34	0.23 ± 0.08	1.90 ± 0.62	1.34 ± 0.23	0.11 ± 0.04	0.80 ± 0.15	0.96 ± 0.19	0.53 ± 0.22	0.45 ± 0.18
NR-E	21.4 ± 2.2	443 ± 94	14956 ± 2759	1.16 ± 0.14	0.24 ± 0.08	2.09 ± 0.67	2.06 ± 0.35	0.12 ± 0.03	1.10 ± 0.32	1.75 ± 0.46	0.50 ± 0.14	0.55 ± 0.15
NR-N	19.5 ± 3.8	467 ± 73	13058 ± 1114	0.94 ± 0.28	0.28 ± 0.08	2.41 ± 0.44	1.90 ± 0.39	0.11 ± 0.03	1.00 ± 0.20	2.12 ± 0.58	0.39 ± 0.07	0.41 ± 0.01
Plant (P)	ns	ns	<0.05	<0.05	ns	ns	<0.01	ns	ns	<0.01	ns	ns
Endophyte (E)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
P x E	ns	ns	ns	ns	ns	<0.05	ns	ns	ns	ns	ns	<0.05
R. acetosa												
R-E	5.1 ± 2.8	136 ± 19	4918 ± 399	0.39 ± 0.15	0.03 ± 0.01	0.38 ± 0.05	0.39 ± 0.15	0.03 ± 0.01	0.38 ± 0.05	0.95 ± 0.14	0.27 ± 0.08	0.29 ± 0.03
R-N	4.3 ± 1.7	107 ± 21	5729 ± 1032	0.38 ± 0.17	0.03 ± 0.00	0.43 ± 0.10	0.38 ± 0.17	0.03 ± 0.00	0.43 ± 0.10	0.88 ± 0.26	0.26 ± 0.07	0.26 ± 0.11
NR-E	3.6 ± 1.0	115 ± 31	6474 ± 670	0.34 ± 0.12	0.03 ± 0.00	0.47 ± 0.07	0.34 ± 0.12	0.03 ± 0.00	0.47 ± 0.07	1.13 ± 0.27	0.27 ± 0.17	0.24 ± 0.07
NR-N	3.4 ± 1.2	93 ± 20	5523 ± 1278	0.33 ± 0.13	0.02 ± 0.01	0.41 ± 0.11	0.33 ± 0.13	0.02 ± 0.01	0.41 ± 0.11	1.15 ± 0.24	0.22 ± 0.10	0.21 ± 0.02
Plant (P)	ns	ns	ns	<0.05	ns	<0.05	ns	ns	ns	ns	ns	ns
Endophyte (E)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
P x E	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Concentrations of Cd, Pb and Zn in plant tissues are shown in Table 5.3. In general, NR pots showed higher values of (i) metal concentration in *N. caerulescens* plants, as reflected by the higher shoot Zn concentrations, (ii) the Cd bioaccumulation factor-BAF (*i.e.*, metal concentration in shoots divided by total metal concentration in soil) and (iii) the Cd bioconcentration factor-BCF (*i.e.*, metal concentration in roots divided by total metal concentration in soil). On the other hand, lower values of the Cd translocation factor-TF (*i.e.*, metal concentration in shoots divided by metal concentration in roots, TF) were observed in NR pots. The interaction between "plant species" and "endophyte inoculation" was significant for TF and BCF of Zn; then, the Fisher's LSD-test indicated that these two parameters were significantly higher in endophyte-inoculated *N. caerulescens* plants (N-E treatment), compared to *N. caerulescens* plants without putative endophyte inoculation (N-N treatment). Pertaining to *R. acetosa* plants, no significant differences in shoot metal concentrations, BAFs or BCFs were observed; likewise, the "plant species" factor only had a significant effect on TF values, with NR pots showing higher TF values for Zn and lower TF values for Cd.

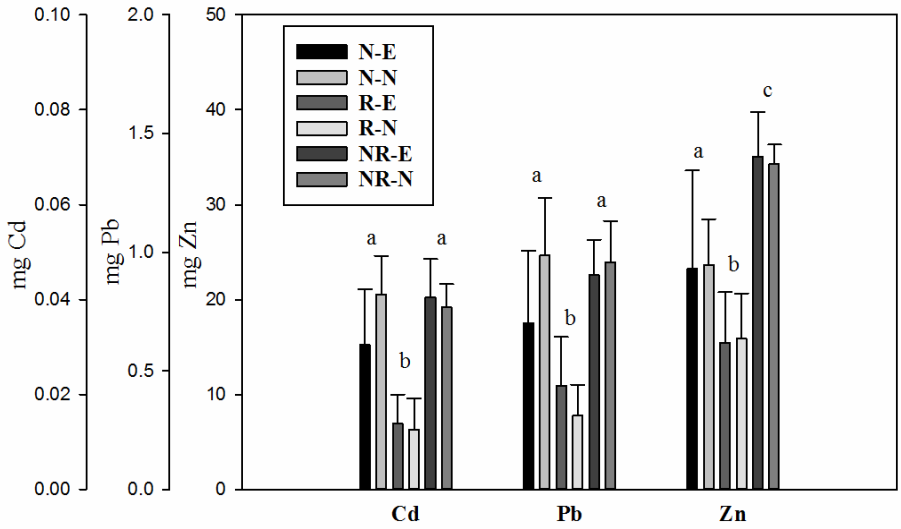


Figure 5.2. Effect of treatments on the amount of metal phytoextracted (mg). Mean values (n = 4) ± SD. Treatments with different letters are significantly (P<0.05) different according to Fisher's LSD-test. Probability values from two-way ANOVA (ns: non-significant; *, ** and *** represent significances of P<0.05, 0.01 and 0.001, respectively) for Cd, Pb and Zn: plant, ***, ***, ***; endophyte, ns, ns, ns; plant x endophyte, ns, ns, ns. N-E: four plants of *N. caerulescens* inoculated with the endophyte consortium; N-N: four plants of *N. caerulescens*, without endophyte inoculation; R-E: four plants of *R. acetosa* inoculated with the endophyte consortium; R-N: four plants of *R. acetosa*, without endophyte inoculation; NR-E: two plants of *N. caerulescens* + two plants of *R. acetosa*, inoculated with both endophyte consortia combined together; NR-N: two plants of *N. caerulescens* + two plants of *R. acetosa*, without endophyte inoculation; UP: unplanted.

Regarding the effects of the experimental factors on total amount of metal phytoextracted, only the effect of "plant species" was statistically significant. Based on this effect, Fisher's LSD-test was used to establish the significance of the differences among means (Figure 5.2). The amount of Cd and Pb phytoextracted was significantly higher in N and NR pots, compared to R pots; in turn, the highest amount of Zn phytoextracted was found in NR pots, followed by N pots.

5.3.3 Effects on soil parameters

According to ANOVA, NR pots had lower values of total Cd concentration in soil, compared to unplanted controls (Table 5.4). Regarding the bioavailable metal fraction, N pots showed the lowest values of $CaCl_2$-extractable concentrations of Cd, Pb and Zn, followed by NR and R pots. In relation to the values of relative metal bioavailability in soil (*i.e.*, $CaCl_2$-extractable metal concentration divided by total metal concentration), N pots showed the lowest values of relative Cd bioavailability. Endophyte inoculation seemed to have a significant effect on relative Cd bioavailability (due to increased values of $CaCl_2$-extractable Cd concentration).

Regarding soil microbial properties, N and R pots appeared to have increased values of most microbial activity parameters (Figure 5.3a; $F = 7.1$; $P<0.01$). Similarly, endophyte inoculation seemed to positively influence microbial parameters (*i.e.*, their values increased along the first axis). However, according to the variation partitioning analysis, the "plant species" factor accounted for 26% of the explained variation, while "endophyte inoculation" explained only 6% of variation. But the short length of most arrows indicates that there was no correlation between these microbial parameters and the different treatments. The ANOVA indicated that "plant species" had a significant effect on values of FDA, urease activity, AWCD and basal respiration, while "endophyte inoculation" had a significant effect on acid phosphatase activity (Supplementary Table 5.1). According to the variation partitioning and RDA analyses applied to microbial biomass parameters (Figure 5.3b; $F = 18.3$; $P<0.01$), the "plant species" factor explained 43% of the variation, with NR pots showing higher values of SIR and bacterial gene abundance (fungal gene abundance was higher in N pots). "Endophyte inoculation", which explained 11% of the variation, resulted in higher values of both bacterial and fungal gene abundance, as corroborated by the significant effects found by ANOVA (Supplementary Table 5.2). Figure 5.4a ($F = 5.5$, $P<0.01$) and Figure 5.4b ($F = 5.1$, $P<0.01$) show RDAs of community-level genetic profiles

Table 5.4. Effect of treatments on total and $CaCl_2$-extractable metal concentrations in soil (mg kg^{-1} DW) and relative metal bioavailability ([metal]bioavailable/[metal]total). Mean values (n = 4) ± SD. Probability values from two-way ANOVA (ns: non-significant) for the effects of "plant species" and "endophyte inoculation" and their interactions are shown below. N-E: four plants of *N. caerulescens* inoculated with the endophyte consortium; N-N: four plants of *N. caerulescens*, without endophyte inoculation; R-E: four plants of *R. acetosa* inoculated with the endophyte consortium; R-N: four plants of *R. acetosa*, without endophyte inoculation; NR-E: two plants of *N. caerulescens* + two plants of *R. acetosa*, inoculated with both endophyte consortia combined together; NR-N: two plants of *N. caerulescens* + two plants of *R. acetosa*, without endophyte inoculation; UP: unplanted.

	Total			**$CaCl_2$-extractable**			**Relative Bioavailability**		
	Cd	**Pb**	**Zn**	**Cd**	**Pb**	**Zn**	**Cd**	**Pb**	**Zn**
N-E	11.2 ± 0.9	3583 ± 277	14446 ± 2736	1.85 ± 0.04	5.66 ± 0.10	454 ± 25	16.6 ± 1.5	0.16 ± 0.01	3.27 ± 0.89
N-N	13.4 ± 0.8	3931 ± 615	13047 ± 1844	1.80 ± 0.07	5.90 ± 0.69	457 ± 22	13.5 ± 1.2	0.15 ± 0.03	3.56 ± 0.58
R-E	11.7 ± 1.4	4301 ± 550	13095 ± 696	1.94 ± 0.05	7.34 ± 0.34	481 ± 9	16.6 ± 1.6	0.17 ± 0.02	3.69 ± 0.18
R-N	11.8 ± 1.3	3873 ± 443	13561 ± 1840	1.86 ± 0.08	6.71 ± 0.82	489 ± 21	15.9 ± 1.5	0.18 ± 0.04	3.65 ± 0.40
NR-E	10.8 ± 1.3	3957 ± 911	13825 ± 1562	1.89 ± 0.08	6.76 ± 0.62	502 ± 9	17.6 ± 2.2	0.18 ± 0.04	3.68 ± 0.50
NR-N	10.3 ± 0.6	4462 ± 923	13390 ± 2253	1.86 ± 0.04	6.46 ± 0.52	497 ± 13	18.1 ± 1.3	0.15 ± 0.04	3.79 ± 0.56
UP	11.5 ± 1.2	4275 ± 563	14425 ± 1460	1.96 ± 0.03	6.98 ± 0.29	517 ± 22	17.2 ± 1.9	0.17 ± 0.02	3.61 ± 0.31
Plant (P)	<0.05	ns	ns	<0.01	<0.01	<0.01	<0.05	ns	ns
Endophyte (E)	ns	ns	ns	<0.05	ns	ns	ns	ns	ns
P x E	ns	ns	ns	ns	ns	ns	ns	ns	ns

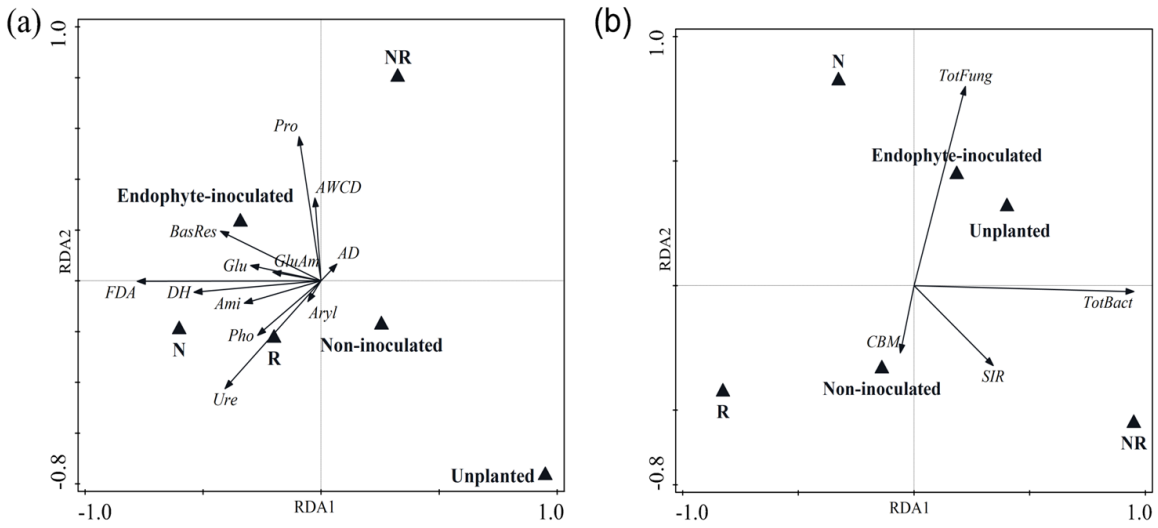


Figure 5.3. Biplots of the redundancy analysis (RDA) performed on: (a) microbial activity parameters; RDA1 and RDA2 account for 51 and 2% of the variance, respectively; (b) microbial biomass parameters; RDA1 and RDA2 account for 62 and 13% of the variance, respectively. Pho: phosphatase; Glu: β-glucosidase; GluAm: β-glucosaminidase; Aryl: arylsulphatase; DH: dehydrogenase; FDA: fluorescein diacetate hydrolysis; Ure: urease; AD: arginine deaminase; Ami: amidase; Pro: protease; AWCD: average well colour development; MBC: microbial biomass carbon; BasRes: basal respiration; SIR: substrate-induced respiration; Tot: total gene abundance; Bact: bacteria; Fung: fungi. Closed symbols represent the different levels of the experimental factors.

discriminating among the "plant species" factor: according to the variation partitioning analysis, "plant species" appeared to be the factor with the largest effect on the diversity of bacterial and fungal communities, with 21 and 27% of explained variation, respectively. On the contrary, "endophyte inoculation" explained only 6% of the variation of bacterial diversity and had no significant effect on fungal diversity. Finally, the RDA of community-level physiological profiles (Figure 5.4c; F = 3.8, P<0.01) suggested a correlation between NR treatment and some of the utilised carbon substrates, indicating a positive influence of this treatment on microbial functional diversity, as corroborated by ANOVA (Supplementary Table 5.2). Variation partitioning analysis states that "plant species" explained 26% of the variation, while "endophyte inoculation" was not statistically significant.

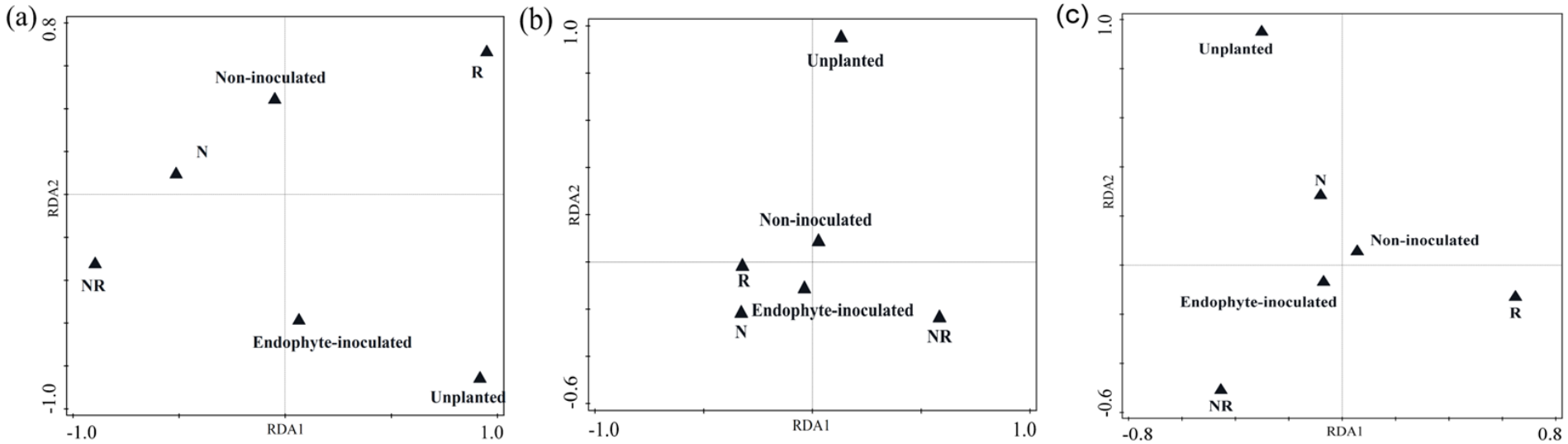


Figure 5.4. Biplots of the redundancy analysis (RDA) performed on: (a) bacterial ARISA community profiles; RDA1 and RDA2 account for 40 and 4% of the variance, respectively; (b) fungal ARISA community profiles; RDA1 and RDA2 account for 30 and 7% of the variance, respectively; (c) Biolog EcoPlates™ physiological profiles; RDA1 and RDA2 account for 31 and 8% of the variance, respectively. Closed symbols represent the different levels of the experimental factors.

5.3.4 Effects on ecosystem services

The overall effect of treatments on ecosystem services, calculated from data on soil parameters, is presented in Figure 5.5, which shows the positive effect of plant growth on the majority of ecosystem services, compared to unplanted controls (UP). NR pots showed higher values of the following ecosystem services: *soil biodiversity*, *water flow regulation* and *pollution control*. N and R treatments also resulted in significantly higher values of ecosystem services: N treatment increased *nutrient cycling*, *water flow regulation*, *water purification* and *contamination control*; R treatment increased *nutrient cycling*. On the other hand, values of *nutrient cycling* in endophyte-inoculated pots were significantly higher than in non-inoculated pots, according to ANOVA (Table 5.5). Finally, according to the Soil Quality Index (SQI), plant growth had a significant positive effect on soil quality.

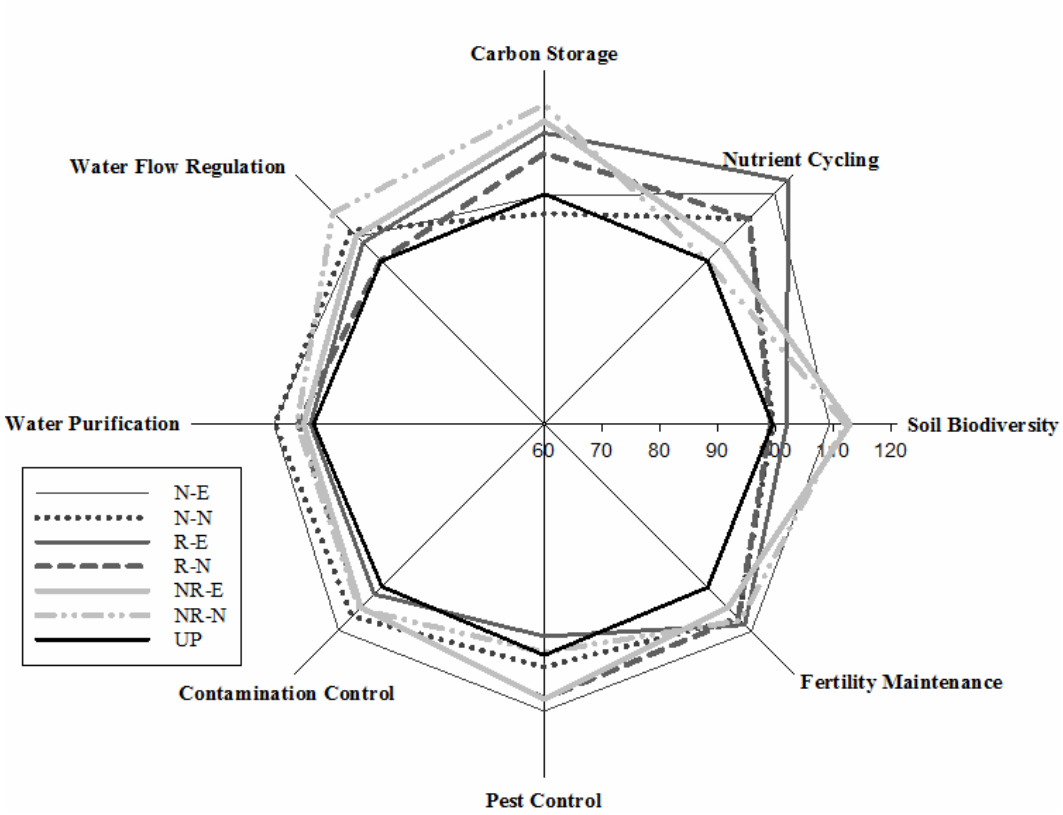


Figure 5.5. Sunray plot of ecosystem services. A value of 100 corresponds to the mean value obtained for each ecosystem service in the unplanted (UP) control treatment. N-E: four plants of *N. caerulescens* inoculated with the endophyte consortium; N-N: four plants of *N. caerulescens*, without endophyte inoculation; R-E: four plants of *R. acetosa* inoculated with the endophyte consortium; R-N: four plants of *R. acetosa*, without endophyte inoculation; NR-E: two plants of *N. caerulescens* + two plants of *R. acetosa*, inoculated with both endophyte consortia combined together; NR-N: two plants of *N. caerulescens* + two plants of *R. acetosa*, without endophyte inoculation; UP: unplanted.

Table 5.5. Effect of treatments on ecosystem services and the soil quality index (SQI). Values are expressed as a percentage of the unplanted control (UP). Mean values (n = 4) ± SD. Probability values from two-way ANOVA (ns: non-significant) for the effects of "plant species" and "endophyte inoculation" and their interactions are shown below. N-E: four plants of *N. caerulescens* inoculated with the endophyte consortium; N-N: four plants of *N. caerulescens*, without endophyte inoculation; R-E: four plants of *R. acetosa* inoculated with the endophyte consortium; R-N: four plants of *R. acetosa*, without endophyte inoculation; NR-E: two plants of *N. caerulescens* + two plants of *R. acetosa*, inoculated with both endophyte consortia combined together; NR-N: two plants of *N. caerulescens* + two plants of *R. acetosa*, without endophyte inoculation; UP: unplanted.

	Soil Biodiversity	Nutrient Cycling	Carbon Storage	Water Flow Regulation	Water Purification	Contamination Control	Pest Control	Fertility Maintenance	SQI
N-E	109 ± 8	116 ± 3	100 ± 2	106 ± 3	107 ± 2	111 ± 4	110 ± 18	111 ± 6	109 ± 4
N-N	100 ± 2	110 ± 7	97 ± 4	107 ± 2	107 ± 3	107 ± 5	102 ± 20	108 ± 8	105 ± 4
R-E	102 ± 5	120 ± 3	110 ± 8	104 ± 5	101 ± 2	102 ± 6	97 ± 10	109 ± 7	106 ± 2
R-N	99 ± 7	110 ± 8	107 ± 14	100 ± 6	103 ± 3	105 ± 3	108 ± 4	108 ± 8	105 ± 2
NR-E	113 ± 8	104 ± 4	113 ± 15	106 ± 4	102 ± 2	105 ± 4	108 ± 30	105 ± 10	107 ± 5
NR-N	110 ± 1	100 ± 3	115 ± 22	112 ± 2	103 ± 2	106 ± 2	99 ± 16	108 ± 6	107 ± 4
UP	100 ± 5	100 ± 3	100 ± 6	100 ± 3	100 ± 1	100 ± 4	100 ± 4	100 ± 5	100 ± 2
Plant (P)	<0.05	<0.001	ns	<0.01	<0.001	<0.05	ns	ns	<0.05
Endophyte (E)	ns	<0.01	ns	ns	ns	ns	ns	ns	ns
P x E	ns	ns	ns	ns	ns	ns	ns	ns	ns

5.4 Discussion

5.4.1 Isolation and characterization of bacterial putative endophytes

As a result of their particular characteristics (*i.e*., poor physical structure, high concentrations of heavy metals, and deficiency of nutrients), mining areas frequently harbour a unique assemblage of plant species and associated microbial communities (showing different metal tolerance and accumulation patterns) with great potential for the phytoremediation and phytomanagement of soils contaminated with high concentrations of heavy metals (Barrutia et al., 2011). In this study, putative endophytic bacterial strains were isolated from two metallophyte species (hyperaccumulator: *N. caerulescens*; accumulator: *R. acetosa*) from an abandoned mine. From the 31 putative endophytic bacterial strains isolated from the interior tissues of *N. caerulescens* and *R. acetosa*, a total of 12 isolates contained ACC deaminase activity. These 12 isolates were then tested for other plant growth-promoting traits (Table 5.1), including IAA production, phosphate solubilisation and siderophore production, that can have a beneficial effect on plant hosts (Rajkumar et al., 2009). Based on these traits, 6 isolates (3 from each metallophyte species) were selected for endophyte-assisted phytoextraction. The 6 selected strains belonged to the genera *Microbacterium*, *Variovorax*, *Micrococcus* and *Pseudomonas*, which have already been associated with endophytic bacteria (Xinxian et al., 2011).

5.4.2 Plant growth and physiological status

NR pots showed higher values of chlorophyll and carotenoid contents, as well as a higher photochemical efficiency, in *N. caerulescens* plants by an average of 35, 29 and 34%, respectively (Table 5.2), indicating a beneficial effect on the plants´ physiological status. On the contrary, NR treatment resulted in lower contents of chlorophylls and carotenoids in *R. acetosa*. But regardless of the effects on plant physiological parameters, and in accordance with other experiments carried out with metal hyperaccumulators (Yang et al., 2007; Epelde et al., 2012b; Lucisine et al., 2014), the simultaneous presence of both species (NR) stimulated plant growth, particularly aerial parts, increasing the total plant biomass of *N. caerulescens* and *R. acetosa* by 41 and 16%, respectively, compared to N and R treatments. These results suggest that an increment in plant growth is not necessarily reflected in an improvement of the plants´ physiological parameters.

Endophyte-inoculated plants of *N. caerulescens* and *R. acetosa* showed up to 27 and 28% increase in chlorophyll content, and up to 36 and 23% increase in carotenoid content, respectively, compared to non-inoculated plants. Besides, values of α-tocopherol content were considerably lower in endophyte-inoculated plants, particularly in NR pots. Bearing in mind that high levels of α-tocopherol, along with a low chlorophyll content, have been related to oxidative damage caused by metal-induced stress (Artetxe et al., 2002), our results indicate a reduction in the stress level of plants due to endophyte-inoculation. This might have resulted from ACC deaminase activity leading to a reduction of stress-induced ethylene levels in plants (Glick, 2014).

5.4.3 Phytoextraction assisted with putative endophytic bacteria

In all treatments, Zn concentration in *N. caerulescens* shoots exceeded the 10,000 mg kg^{-1} DW threshold set for Zn hyperaccumulators (Baker et al., 2000) (Table 5.3). However, highest values of shoot metal concentration and BAF (BAF is considered to be more important than shoot metal concentration when evaluating phytoextraction potential) (Barrutia et al., 2009) were found for Zn in NR pots, highlighting the relationship between co-planting (NR treatment) and the increase in metal uptake by *N. caerulescens*. This goes in accordance with several experiments which demonstrated that certain metallophytes tend to accumulate more metals under plant species coexistence (Liu et al., 2011; Epelde et al., 2012b; Yang et al., 2012). Indeed, the highest quantity of Zn phytoextracted was found in NR pots, which corroborates that the combination of *N. caerulescens* and *R. acetosa* is a most suitable option for Zn phytoextraction (Epelde et al., 2012b).

The lower TF values found for Cd in *N. caerulescens* plants in NR pots indicate that this metal was not completely translocated to the aerial parts. Indeed, BCF values, which are inversely correlated to TF values, suggested that heavy metals were accumulating in the root tissues. Lucisine et al. (2014) observed a delayed translocation of heavy metals in hyperaccumulators growing under plant species coexistence, which could partly explain the moderate translocation of Cd observed here. Our data *a priori* suggest the possibility of using these plant species for Cd (and even Pb) phytostabilization, but the high shoot Zn concentrations observed here and the relatively low BCFs discourage that option. Both BAF and BCF values obtained in this study are relatively low, most probably due to the elevated soil metal concentrations.

Since metal bioavailability is a key factor limiting metal uptake by plants, strategies to increase metal bioavailability in soil can be used to optimize phytoextraction. Thus, Liu et al. (2011) reported that the use of co-planting increased metal bioavailability in soil, resulting in higher values of metal accumulation by plants. In our study, metal concentrations in soil were fairly similar for every treatment (Table 5.4). The presence of any plant, and particularly *N. caerulescens*, resulted in lower $CaCl_2$-extractable metal concentrations, compared to unplanted controls (Table 5.4). This may be due to an increase in metal uptake by plants, leading to a smaller pool of bioavailable metals in soil.

On the other hand, endophyte-inoculated pots showed a higher bioavailable fraction of Cd in soil than non-inoculated pots. Some studies have demonstrated that endophytes can increase heavy metal bioavailability in soil, probably through the release of metals from non-soluble phases, thus facilitating phytoextraction (Mastretta et al., 2009; Rajkumar et al., 2009; Chen et al., 2010). Our results suggest that endophyte inoculation might have facilitated the translocation of Zn in *N. caerulescens* plants among N pots, which concomitantly resulted in lower BCF values. Endophytes able to naturally produce metal chelators, such as siderophores, have been reported to promote translocation of heavy metals from roots to aerial parts (Mastretta et al., 2009; Luo et al., 2011).

5.4.4 Soil microbial properties

Chemical data alone are not sufficient to properly estimate the effectiveness of phytoremediation, in terms of the recovery of soil functioning. Microbial properties have been recommended as indicators of the functioning of the soil ecosystem in metal contaminated and phytoremediated soils (Gómez-Sagasti et al., 2012).

Yang et al. (2012) demonstrated that co-planting of multiple plant species increased soil microbial activity; however, and similarly to previous studies (Epelde et al., 2012b), we found here that co-planting of metallophytes and soil enzyme activities were negatively correlated. Microbial biomass, in contrast, was positively influenced by co-planting, as reflected in the higher values of SIR and bacterial gene abundance. Moreover, community-level genetic profiles of bacterial and fungal communities strongly discriminated among the “plant species” factor, indicating differences in microbial composition due to plant composition. These differences in microbial biomass and diversity may result from differences in the amount and quality of root exudates

(Lucisine et al., 2014). At the same time, a higher diversity of root exudates may induce shifts of substrate utilization patterns by the soil microbial communities, increasing functional diversity (Yang et al., 2007), as reflected here by the higher values of NUS from CLPPs (Supplementary Table 5.2).

With regard to the effects of endophytes, acid phosphatase activity was the only microbial activity parameter that showed statistically significant differences due to endophyte inoculation. On the other hand, higher values of bacterial and fungal abundance were observed in endophyte-inoculated pots, suggesting a stimulation of microbial growth by endophytes. Bacterial ARISA community profiles also indicated effects on the composition of soil bacterial communities (Figure 5.4a). Chen et al. (2013) demonstrated that plant growth-promoting bacteria can change the bacterial community structure of rhizosphere and bulk soil, by inducing proliferation of other rhizosphere bacteria on the root surfaces. This stimulation of microbial growth may be due to the release of specific sugars and amino acids into the rhizosphere by plants (Epelde et al., 2010b), which could be facilitated as a result of changes in their physiological status due to endophyte inoculation.

5.4.5 Evaluation through ecosystem services

As already stated, the recovery of ecosystem services during phytoremediation can be considered for a better interpretation of results, by linking the concept of soil quality to that of ecosystem services. For this purpose, we grouped soil properties (particularly soil microbial parameters) within a set of ecosystem services. According to our results, *N. caerulescens* growth improved the following ecosystem services: *water flow regulation*, *water purification* and *contamination control* (Table 5.5). Even though a high metal bioavailability in soil is highly desirable to facilitate metal phytoextraction (Xinxian et al., 2011), metals in their bioavailable form are more toxic, compromising the functioning of the soil ecosystem and posing a major threat to human health. Consequently, the lower metal bioavailability found in the presence of *N. caerulescens* plants was reflected in an improvement of these ecosystem services, particularly under co-planting. This confirms the superiority of *N. caerulescens* for phytoextraction in terms of the recovery of soil functioning (*N. caerulescens* has greater potential for hyperaccumulation than *R. acetosa*) (Barrutia et al., 2009).

Pertaining to ecosystem services related to microbial properties, values of *nutrient cycling* corroborated the negative correlation between co-planting and

microbial activity parameters, whereas increased values of this ecosystem service were found in N and R pots. In contrast, co-planting resulted in higher values of *soil biodiversity*. Since different plant species can be associated with different microbial communities, co-planting may enhance the complexity of soil microbial communities resulting in a higher functional diversity (Yang et al., 2007; Lucisine et al., 2014). However, we previously found that plant biomass can have a stronger effect on soil microbial parameters than plant diversity (Epelde et al., 2010b); then, the larger plant biomass obtained in NR pots could also explain the increase in microbial diversity.

The main ecosystem service improvement observed here, as a consequence of putative endophyte inoculation, appears to be *nutrient cycling*. As aforementioned, putative endophyte inoculation might have contributed to a reduction in plant stress levels, which, in turn, might have had a positive effect on the development of rhizobacteria through stimulated root exudation by plants, finally resulting in an enhancement of soil *nutrient cycling*.

The SQI, calculated from the values of the different ecosystem services estimated here, corroborated the positive effect of *N. caerulescens* and *R. acetosa* growth on soil properties (*i.e.*, increase in soil microbial activity, biomass and diversity) and ecosystem services (*i.e.*, increase in soil biodiversity, nutrient cycling, water flow regulation, water purification and contamination control); however, no significant differences are observed among the different plant treatments (N, R, NR). SQI values must be interpreted with caution: the SQI is a simplified reflection of the exceptionally complex soil ecosystem and, inexorably, it entails a certain loss of information regarding the response of each parameter considered during the calculation of the SQI (Burges et al., 2015).

As previously reported (Burges et al., 2016), the interpretation of data on ecosystem services led here to similar conclusions to those directly obtained from data on soil properties. Nevertheless, for a complementary interpretation of results, it is useful to group soil properties (*i.e.*, soil quality indicators) into ecosystem services (Gómez-Sagasti et al., 2012). The main advantage of this approach relies on the fact that these higher, more-general categories are easier to interpret and should be *a priori* much less affected by changes in techniques, methods, equipment, interests, etc., which might occur during long-term monitoring programs (Epelde et al., 2014a).

5.5 Conclusions

Co-planting of *N. caerulescens* and *R. acetosa* stimulated plant growth and enhanced metal phytoextraction, resulting in a reduction of metal bioavailability and an increase in soil microbial biomass and functional diversity. Our results highlight the suitability of co-planting of metallophytes for phytoextraction. On the other hand, putative endophyte inoculation improved the plants´ physiological status, providing them with fitness to counter the problems associated to plant growth in mine soil and, then, resulting in an improvement in some indicators of soil quality. The evaluation of soil quality through the grouping of soil properties within ecosystem services has proved to be a valid tool to assess the efficiency of phytoextraction.

Supplementary Table 5.1. Effect of treatments on soil microbial activity and biomass. Mean values (n = 4) ± SD. Probability values from two-way ANOVA (ns: non-significant) for the effects of "plant species" and "endophyte inoculation" and their interactions are shown below. N-E: *N. caerulescens*, inoculated with endophyte; N-E: four plants of *N. caerulescens* inoculated with the endophyte consortium; N-N: four plants of *N. caerulescens*, without endophyte inoculation; R-E: four plants of *R. acetosa* inoculated with the endophyte consortium; R-N: four plants of *R. acetosa*, without endophyte inoculation; NR-E: two plants of *N. caerulescens* + two plants of *R. acetosa*, inoculated with both endophyte consortia combined together; NR-N: two plants of *N. caerulescens* + two plants of *R. acetosa*, without endophyte inoculation; UP: unplanted.

	Pho	Glu	GluAm	Aryl	DH	FDA	Ure	AD	Ami	Pro	BasRes	AWCD	MBC	SIR	Bact	Fung
N-E	196 ± 13	61 ± 6	43 ± 5	57 ± 6	9.8 ± 0.9	468 ± 69	5.1 ± 1.8	1.2 ± 0.2	158 ±16	316 ± 14	0.66 ± 0.13	0.18 ± 0.02	86 ± 6	2.9 ± 0.4	318 ± 38	2.5 ± 0.1
N-N	188 ± 6	55 ± 9	48 ± 8	56 ± 7	9.0 ± 0.5	474 ± 34	2.8 ± 1.7	1.3 ± 0.2	158 ± 7	306 ± 20	0.78 ± 0.10	0.15 ± 0.04	82 ± 2	2.6 ± 0.8	244 ± 58	1.9 ± 0.3
R-E	195 ± 21	65 ± 3	45 ± 10	65 ± 11	8.2 ± 1.9	467 ± 54	6.3 ± 2.5	1.3 ± 0.2	175 ± 7	301 ± 39	0.62 ± 0.05	0.14 ± 0.02	92 ± 12	3.8 ± 0.3	256 ± 38	1.7 ± 0.2
R-N	184 ± 16	64 ± 3	44 ± 7	66 ± 7	9.0 ± 0.7	386 ± 42	5.0 ± 2.3	1.2 ± 0.3	152 ± 16	281 ± 50	0.55 ± 0.11	0.12 ± 0.02	96 ± 9	3.4 ± 1.0	160 ± 30	1.1 ± 0.2
NR-E	194 ± 2	60 ± 10	39 ± 19	62 ± 5	8.9 ± 0.5	362 ± 79	1.8 ± 0.7	1.4 ± 0.1	147 ± 13	314 ± 48	0.63 ± 0.17	0.16 ± 0.02	93 ± 13	4.4 ± 1.7	700 ± 68	1.6 ± 0.1
NR-N	167 ± 12	53 ± 13	41 ± 7	55 ± 9	8.5 ± 0.6	351 ± 53	2.6 ± 0.7	1.2 ± 0.1	153 ± 22	328 ± 16	0.60 ± 0.14	0.18± 0.01	88 ± 7	5.3 ± 2.6	611 ± 103	1.4 ± 0.2
UP	192 ± 11	53 ± 9	40 ± 3	64 ± 13	8.5 ± 0.3	303 ± 47	3.9 ± 1.3	1.3 ± 0.2	148 ± 20	306 ± 31	0.52 ± 0.04	0.15 ± 0.02	89 ± 11	4.3 ± 1.4	465 ± 80	1.7 ± 0.1
Plant (P)	ns	ns	ns	ns	ns	<0.01	<0.01	ns	ns	ns	<0.05	<0.05	ns	<0.05	<0.001	<0.001
Endophyte (E)	<0.01	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	<0.01	<0.001
P x E	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	<0.01

Pho: phosphatase; Glu: β-glucosidase; GluAm: β-glucosaminidase; Aryl: arylsulphatase (mg ρ-nitrophenol kg^{-1} h^{-1}); DH: dehydrogenase (mg INTF kg^{-1} $20h^{-1}$); FDA: fluorescein diacetate hydrolisis (µg fluorescein g^{-1} h^{-1}); Ure: urease; AD: arginine deaminase (mg $N\text{-}NH_4^+$ kg^{-1} h^{-1}); Ami: amidase (mg β-naphthylamine kg^{-1} h^{-1}); Pro: protease (mg tyrosine kg^{-1} h^{-1}); BasRes: basal respiration; AWCD: average well colour development; MCB: microbial biomass carbon (mg C kg^{-1} DW soil); SIR: substrate-induced respiration (µg C g^{-1} soil h^{-1}); Bact: total bacterial gene abundance; Fung: total fungal gene abundance (x 10^5 copies g^{-1} DW soil).

Supplementary Table 5.2. Effect of treatments on soil microbial diversity. Mean values (n = 4) ± SD. Probability values from two-way ANOVA (ns: non-significant) for the effects of "plant species" and "endophyte inoculation" and their interactions are shown below. N-E: four plants of *N. caerulescens* inoculated with the endophyte consortium; N-N: four plants of *N. caerulescens*, without endophyte inoculation; R-E: four plants of *R. acetosa* inoculated with the endophyte consortium; R-N: four plants of *R. acetosa*, without endophyte inoculation; NR-E: two plants of *N. caerulescens* + two plants of *R. acetosa*, inoculated with both endophyte consortia combined together; NR-N: two plants of *N. caerulescens* + two plants of *R. acetosa*, without endophyte inoculation; UP: unplanted.

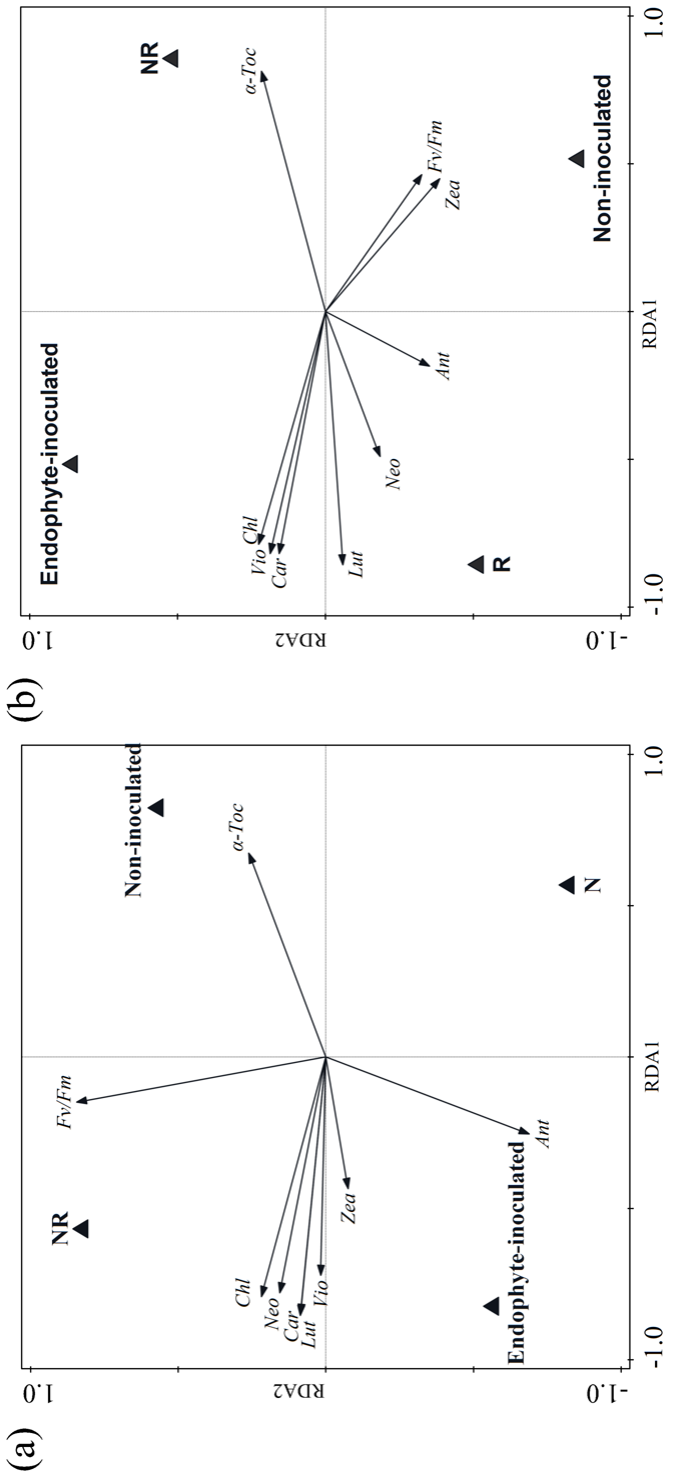
	Biolog EcoPlates™			**ARISA**					
	NUS	**H'**	**J**	**S Bact**	**H' Bact**	**J Bact**	**S Fung**	**H' Fung**	**J Fung**
N-E	6.8 ± 1.0	2.3 ± 0.3	0.83 ± 0.04	25 ± 3	4.2 ± 0.2	0.90 ± 0.01	9 ± 1	2.3 ± 0.2	0.70 ± 0.02
N-N	5.4 ± 1.1	2.0 ± 0.3	0.81 ± 0.04	24 ± 2	4.0 ± 0.1	0.88 ± 0.02	8 ± 2	2.2 ± 0.4	0.71 ± 0.09
R-E	4.3 ± 1.2	2.2 ± 0.4	1.05 ± 0.20	27 ± 4	4.2 ± 0.2	0.88 ± 0.01	13 ± 2	2.2 ± 0.4	0.62 ± 0.08
R-N	4.8 ± 1.4	2.0 ± 0.4	0.91 ± 0.09	30 ± 2	4.4 ± 0.1	0.89 ± 0.02	9 ± 1	2.1 ± 0.3	0.63 ± 0.10
NR-E	6.7 ± 1.3	2.3 ± 0.2	0.84 ± 0.12	28 ± 2	4.3 ± 0.1	0.91 ± 0.00	12 ± 1	2.5 ± 0.1	0.71 ± 0.04
NR-N	7.8 ± 1.0	2.3 ± 0.4	0.78 ± 0.12	27 ± 4	4.1 ± 0.2	0.87 ± 0.02	12 ± 1	2.3 ± 0.2	0.69 ± 0.09
UP	5.5 ± 1.0	2.0 ± 0.3	0.81 ± 0.03	26 ± 5	4.1 ± 0.2	0.88 ± 0.00	9 ± 2	1.9 ± 0.3	0.60 ± 0.03
Plant (P)	<0.01	ns	<0.05	ns	ns	ns	<0.01	ns	<0.05
Endophyte (E)	ns	ns	ns	ns	ns	<0.05	<0.05	ns	ns
P x E	ns	ns	ns	ns	ns	<0.01	ns	ns	ns

NUS: number of utilised substrates; H': Shannon's diversity; S: richness; J': Pielou's evenness; Bact: bacteria; Fung: fungi.

Supplementary Table 5.3. Effect of treatments on physicochemical parameters and suppressiveness. Mean values (n = 4) ± SD. Probability values from two-way ANOVA (ns: non-significant) for the effects of "plant species" and "endophyte inoculation" and their interactions are shown below. N-E: four plants of *N. caerulescens* inoculated with the endophyte consortium; N-N: four plants of *N. caerulescens*, without endophyte inoculation; R-E: four plants of *R. acetosa* inoculated with the endophyte consortium; R-N: four plants of *R. acetosa*, without endophyte inoculation; NR-E: two plants of *N. caerulescens* + two plants of *R. acetosa*, inoculated with both endophyte consortia combined together; NR-N: two plants of *N. caerulescens* + two plants of *R. acetosa*, without endophyte inoculation; UP: unplanted.

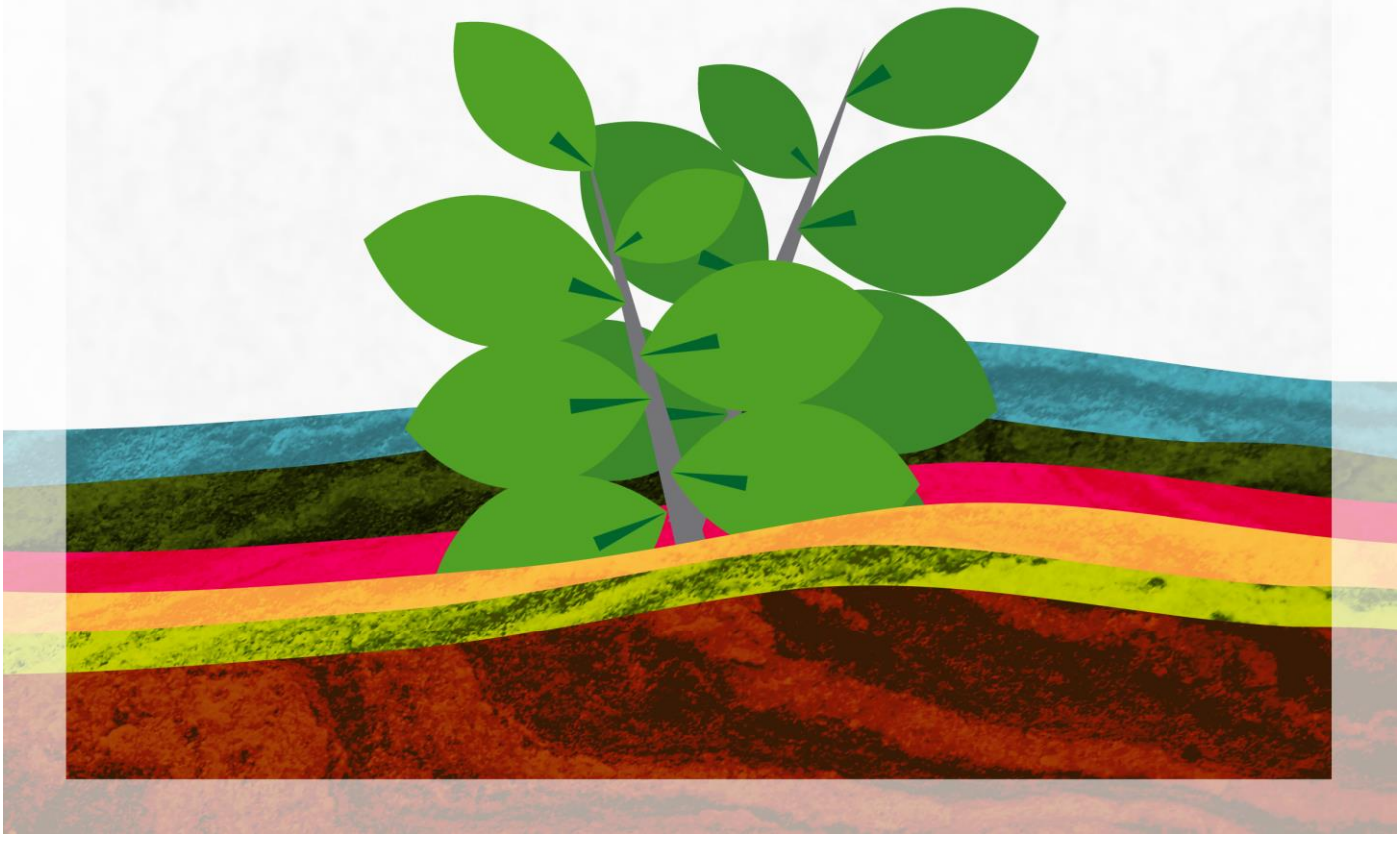
	Total C	**Total N**	**SWC**	**Organic C**	**Suppress**	**Sol Cd**	**Sol Pb**	**Sol Zn**
N-E	3.4 ± 0.2	0.28 ± 0.01	29.8 ± 1.0	66 ± 5	0.49 ± 0.08	<0.25	<2.0	10.3 ± 4.8
N-N	3.4 ± 0.1	0.27 ± 0.01	30.2 ± 0.7	66 ± 7	0.45 ± 0.09	<0.25	<2.0	9.1 ± 3.6
R-E	3.5 ± 0.2	0.29 ± 0.02	29.4 ± 1.5	62 ± 10	0.40 ± 0.04	<0.25	<2.0	11.9 ± 6.7
R-N	3.5 ± 0.2	0.28 ± 0.02	28.2 ± 1.7	62 ± 7	0.48 ± 0.02	<0.25	<2.0	9.0 ± 1.8
NR-E	3.3 ± 0.4	0.27 ± 0.02	29.8 ± 1.0	63 ± 10	0.48 ± 0.13	<0.25	<2.0	6.2 ± 2.7
NR-N	3.2 ± 0.3	0.26 ± 0.02	31.4 ± 0.6	68 ± 5	0.39 ± 0.11	<0.25	<2.0	7.7 ± 4.2
UP	3.4 ± 0.0	0.27 ± 0.01	28.1 ± 0.9	57 ± 5	0.47 ± 0.02	<0.25	<2.0	11.2 ± 9.4
Plant (P)	ns	ns	<0.01	ns	ns	ns	ns	ns
Endophyte (E)	ns	ns	ns	ns	ns	ns	ns	ns
P x E	ns	ns	ns	ns	ns	ns	ns	ns

Total C: total carbon content (%); Total N: total nitrogen content (%); SWC: soil water content at field capacity (%); Organic C: organic carbon content (mg C kg^{-1}); Suppress: soil suppressiveness; Sol: soluble metal concentration of Cd, Pb and Zn (mg l^{-1}).



Supplementary Figure 5.1. Biplots of the redundancy analysis (RDA) from content of lipophilic antioxidants, photoprotective and photosynthetic pigments, and photochemical efficiency of: (a) *N. caerulescens*; RDA1 and RDA2 account for 46 and 13% of the variance, respectively; (b) *R. acetosa*; RDA1 and RDA2 account for 51 and 5% of the variance, respectively. Fv/Fm: photochemical efficiency; Vio: violaxanthin; Ant: antheraxanthin; Zea: zeaxanthin; Lut: lutein; Neo: neoxanthin; Car: carotenoids; Chl: chlorophylls a and b; α-Toc: α-tocopherol. Closed symbols represent the different levels of the experimental factors.

06 | ENHANCEMENT OF ECOSYSTEM SERVICES DURING ENDOPHYTE-ASSISTED AIDED PHYTOSTABILIZATION OF METAL CONTAMINATED MINE SOIL



6. ENHANCEMENT OF ECOSYSTEM SERVICES DURING ENDOPHYTE-ASSISTED AIDED PHYTOSTABILIZATION OF METAL CONTAMINATED MINE SOIL

Burges A, Epelde L, Benito G, Artetxe U, Becerril JM, Garbisu C, 2016. Enhancement of ecosystem services during endophhyte-assisted aided phytostabilization of metal contaminated mine soil. ***Science of the Total Environment*** *562: 480-492.*

Abstract

Endophytic plant growth-promoting bacteria (endophytes) were isolated from a variety of (pseudo)metallophytes growing in an abandoned Zn/Pb mine and then characterized according to their plant growth-promoting traits (i.e. ACC deaminase activity, IAA production, siderophore production, phosphate solubilising capacity, metal and salt tolerance and phenotypic characterization). Initially, under growth chamber conditions, an endophyte-assisted aided phytostabilization study was carried out with *Festuca rubra* plants (native *vs.* commercial variety) inoculated with a *Pseudomonas* sp. isolate and cow slurry as organic amendment. The effect of treatments on soil physicochemical and microbial indicators of soil quality, as well as plant physiological parameters and metal concentrations, was assessed. We performed a complementary interpretation of our data through their grouping within a set of ecosystem services. Although the application of cow slurry had the most pronounced effects on soil quality indicators and ecosystem services, the growth of native *F. rubra* plants reduced soil bioavailability of Cd and Zn by 19 and 22%, respectively, and enhanced several soil microbial parameters. On the other hand, endophyte (*Pseudomonas* sp.) inoculation improved the physiological status of *F. rubra* plants by increasing the content of carotenoids, chlorophylls and Fv/Fm by 69, 65 and 37%, respectively, while also increasing the values of several soil microbial parameters. Finally, a consortium of five endophyte isolates was used for an endophyte-assisted aided phytostabilization field experiment, where lower metal concentrations in native excluder plants were found. Nonetheless, the field inoculation of the endophyte consortium had no effect on the biomass of native plants.

6.1 Introduction

Phytostabilization, i.e. the use of metal tolerant plants to decrease soil metal bioavailability and mobility, can be an effective phytomanagement option for soils highly contaminated with metals such as mine soils (Galende et al., 2014a). However,

plant growth on mine soil is often compromised due to high metal concentrations and nutrient deficiencies (Mendez and Maier, 2008). Then, the incorporation of amendments to mine soil during phytostabilization (aided phytostabilization) has been frequently used to facilitate plant colonization (Alvarenga et al., 2009a, 2009b; Epelde et al., 2009a). Regrettably, the beneficial effect of amendments might be transient (Epelde et al., 2014b). Besides, potential environmental impacts and human health risks derived from the application of amendments, such as groundwater contamination by nitrates or an increase in microbial resistance to antibiotics (Goss et al., 2013; Jorge-Mardomingo et al., 2015), have brought up the necessity to explore alternative possibilities.

Metalliferous environments shelter a unique and highly valuable biodiversity. Thus, pseudometallophytes and metallophytes from mine tailings can be most suitable for phytomanagement of metal contaminated sites (Barrutia et al., 2011). On the other hand, plant growth-promoting rhizobacteria (PGPR) and bacterial endophytes isolated from plants growing in metalliferous environments have successfully demonstrated their potential for phytomanagement, owing to their ability to stimulate plant growth and/or protect plants against metal toxicity through several mechanisms (Mastretta et al., 2006; Barzanti et al., 2007; Chen et al., 2010; Zhang et al., 2011; Babu et al., 2013).

The ultimate goal of any soil metal remediation method must be not only to remove the metals from the soil or to render them harmless, but also to restore soil quality (Epelde et al., 2009a). Soil microbial parameters have great potential as biological indicators of the effectiveness of phytomanagement (Epelde et al., 2009a). But despite their proven value as biological indicators of soil quality, microbial parameters are highly-context dependent and usually difficult to interpret. Consequently, we proposed to group soil microbial parameters in higher-level categories such as “ecological attributes” or “ecosystem services” in order to facilitate interpretation and, most importantly, to provide long-term phytostabilization monitoring programs with the required stability through time against changes in techniques, methods, interests, etc. (Garbisu et al., 2011; Epelde et al., 2014a). Considering that the concept of “ecosystem services” is gaining traction as a way of bridging the scientific-economic-policy making divide (Millennium Ecosystem Assessment, 2005), the abovementioned monitoring programs might be based on ecosystem services, thereby providing the best information for decision-making (Chapman, 2012).

The objective of this study was to assess the effectiveness of endophyte-assisted aided phytostabilization for the phytomanagement of metal contaminated mine soil,

with special emphasis on the enhancement of ecosystem services. To this purpose, the following steps were undertaken: (i) isolation of bacterial endophytes from five native (pseudo)metallophyte species growing in an abandoned Pb/Zn mine; (ii) selection of the bacterial endophyte strains with the best plant growth-promoting traits; (iii) an endophyte-assisted aided phytostabilization study was carried out under growth chamber conditions (with a *Pseudomonas* sp. endophyte strain isolated from native *Festuca rubra* and cow slurry as organic amendment) using native *F. rubra* plants *versus* plants of a commercial variety of *F. rubra*; and (iv) an endophyte-assisted phytostabilization field study was carried out in the abovementioned Pb/Zn mine.

6.2 Materials and methods

6.2.1 Site description and plant sampling

The study was carried out in an abandoned Pb/Zn mine located in the province of Biscay (northern Spain, 43º43´N, 3º26´W), with a temperate Atlantic climate (mean annual rainfall: 1,400 mm y^{-1}; mean annual temperature: 11-15 ºC). Lead and zinc extraction by open cast mining ceased in the late 1970s. The mining area includes open pits, overburden surfaces, tailing dams and degraded zones where the soil contains high levels of Cd, Pb and Zn; for a detailed site description, see Barrutia et al. (2011). Healthy plants of *Festuca rubra* L., *Noccaea caerulescens* J. & C. Presl. and *Rumex acetosa* L., together with plantlets of *Betula alba* Ehrh. and *Salix atrocinerea* Brot., were collected at random from this mining site. Plants were washed extensively to remove any adhering soil, first in tap water and then in 0.01 M EDTA, followed by three rinses with deionized water, and then separated into roots, stems and leaves.

6.2.2 Isolation, characterization and identification of endophytes

Bacterial endophytes were isolated separately from roots, stems and leaves following Surette et al. (2003). Based on their colony morphotype (size, shape and colour), a total of 78 bacterial endophyte strains were isolated. Individual colonies were sub-cultured three times on respective growth medium plates (Surette et al., 2003) and then -80 ºC culture stocks supplemented with 20% (v/v) glycerol were prepared.

Initially, all these 78 strains were tested for their 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity following Penrose and Glick (2003), since the ability of bacteria to promote plant growth has been reported to be closely related to their ACC deaminase activity (Glick, 2014); accordingly, only ACC deaminase activity-

containing strains were tested for other plant growth-promoting traits, i.e. indole-3-acetic acid (IAA) production (Becerra-Castro et al., 2011), siderophore production (Schwyn and Neilands, 1987), and phosphate solubilising activity (Nautiyal, 1999). The tolerance of each isolate to Cd, Pb and Zn was evaluated as described by Long et al. (2011). Maximal tolerable concentrations (MTC) were recorded as the highest metal concentration in which each isolate grew. Salt tolerance was tested using Luria-Bertani (LB) (Sigma-Aldrich, USA) agar plates supplemented with increasing concentrations of NaCl (Rashid et al., 2012). A phenotypic characterization of the isolates was performed using GEN III MicroPlates™ (Biolog Inc., Hayward, USA).

The isolates were identified using 16S rRNA gene sequencing. Total genomic DNA was extracted from each isolate using E.Z.N.A.™ bacterial DNA extraction kit (Omega, USA). Amplification of the 16S rRNA gene sequence was performed with the bacterial universal primers 938F and 1378R (Heuer et al., 1997) as described in Epelde et al. (2014b). Subsequently, amplification products were purified with the MultiScreen HTS PCR 96 kit (Merck MilliPore, USA). Sequencing was carried out by using an automatic sequencer ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, CA, USA) at the Genomic Core Facility SGIker (UPV/EHU, Spain). DNA sequences were analysed with basic sequence alignment BLAST program, run against the database from National Center for Biotechnology Information (NCBI).

6.2.3 Growth chamber study

6.2.3.1 Experimental design

An endophyte-assisted aided phytostabilization experiment with *F. rubra* was performed, under controlled growth chamber conditions, in pots containing soil from the abovementioned mine. The soil was collected from the top layer (0-20 cm) in March 2014 and immediately transported to the laboratory where all visible roots were removed. The soil was air-dried, thoroughly mixed and sieved to <4 mm. Subsamples were sieved to <2 mm for physicochemical characterization according to standard methods (MAPA, 1994). The soil was a sandy-loam with the following physicochemical properties: pH = 6.7; OM (%) = 5.3; total N (%) = 0.23; extractable P (mg kg^{-1} dry weight-DW soil) = 2.2; exchangeable K^+ (mEq 100 g^{-1}) = 1.22; exchangeable Ca^{2+} (mEq 100 g^{-1}) = 2.94; exchangeable Mg^{2+} (mEq 100 g^{-1}) = 1.04; and CEC (mEq 100 g^{-1}) = 5.93. Total metal (Cd, Pb and Zn) concentrations were determined using Atomic Absorption Spectroscopy (AAS) (Spectra AA-250 plus, Varian,

Australia) following aqua regia digestion (Zhao et al., 1994). Total metal concentrations in soil were (mg kg^{-1} DW soil): 10.3, 6,659 and 15,266 for Cd, Pb and Zn, respectively.

One half of the soil (sieved to <4 mm) was amended with fresh cow slurry [dry matter (%) = 8.0; pH = 7.3; OM (%) = 74.2; total N (%) = 0.39; total P (%) = 0.70; and total K^+ (%) = 3.45]. A mixer was used to properly homogenize the amendment-soil mixture (1:8, v/v). Unamended soil was also homogenized in the mixer.

Two different varieties of *F. rubra* were used: (i) a native variety from the mine, termed here "Lanestosa" and (ii) a commercial variety (from Semillas Silvestres S.L., Spain). Explants from the Lanestosa variety were collected from the mine, washed thoroughly with tap water to remove any adhering soil, individually planted on a mixture of perlite and vermiculite (1:3, v/v, moistened with deionized water), and allowed to grow for one month in a growth chamber under the following conditions: 14/10 h light/dark cycle, 20/16 ºC day/night temperature, 70% relative humidity, and a photosynthetic photon flux density of 150 µmol photon m^{-2} s^{-1}. Commercial seeds of *F. rubra* were germinated for one month on a mixture of perlite and vermiculite (1:3; v/v) in a growth chamber under the aforementioned conditions. Equally developed plants of the Lanestosa and the commercial variety were selected for this study.

From the endophyte strains isolated from native *F. rubra* plants, the *Pseudomonas* isolate FS4 was selected for inoculation, as it had the best-performing plant growth-promoting characteristics (Table 6.1). *Pseudomonas* FS4 was grown overnight in LB medium at 28 ºC on a rotary shaker at 125 rpm. Cells were collected by centrifugation (6000 *x g*, 4 ºC), washed twice with sterile PBS, and resuspended in sterile 0.85% KCl to obtain a final inoculum density of 10^9 CFU ml^{-1} (OD_{660} = 1.25). *Festuca rubra* plants (Lanestosa and commercial variety) were soaked for 2 h in this bacterial culture following Chen et al. (2010). Finally, six plants were transferred to study pots containing 2.7 kg DW mine soil. Control (non-inoculated) plants were soaked in sterile 0.85% KCl.

A total of ten treatments were conducted: (1) CUE: commercial *F. rubra*, unamended, inoculated with *Pseudomonas* sp. endophyte; (2) CUN: commercial *F. rubra*, unamended, no endophyte inoculation; (3) CAE: commercial *F. rubra*, amended, inoculated with *Pseudomonas* sp. endophyte; (4) CAN: commercial *F. rubra*, amended, no endophyte inoculation; (5) LUE: Lanestosa *F. rubra*, unamended, inoculated with *Pseudomonas* sp. endophyte; (6) LUN: Lanestosa *F. rubra*, unamended, no endophyte inoculation; (7) LAE: Lanestosa *F. rubra*, amended, inoculated with *Pseudomonas* sp.

endophyte; (8) LAN: Lanestosa *F. rubra*, amended, no endophyte inoculation; (9) UPU: unplanted, unamended; and (10) UPA: unplanted, amended. Each treatment was performed in three replicates. Plants were allowed to grow in a growth chamber at the abovementioned conditions and were bottom-watered as needed throughout the experimental period.

6.2.3.2 Plant parameters

After 8 weeks of growth, plants were kept in the dark for 12 h to reduce the effects of diurnal variations on antioxidant and pigment composition and to provide comparable "artificial predawn conditions" (Tausz et al., 2003). Photochemical efficiency (Fv/Fm) was measured with a modulated fluorometer (Fluorpen FP100, Photon Systems Instruments, Drasov, Czech Republic). Then, 0.02 g FW leaf were collected, frozen in liquid nitrogen and stored at -80 ºC until analysis. Lipophilic antioxidants [α-tocopherol (α-Toc)], photoprotective pigments [carotenoids (Car), neoxanthin (Neo), violaxanthin (Vio), antheraxanthin (Ant), zeaxanthin (Zea) and lutein (Lut)] and photosynthetic pigments [chlorophylls a and b (Chl)] were measured by reverse-phase HPLC following García-Plazaola and Becerril (2001).

Shoots and roots were harvested separately, washed thoroughly with deionized water, and oven-dried at 70 ºC for 48 h to calculate dry weights (DW). Subsamples (0.2 g DW) were digested with a mixture of HNO_3/$HClO_4$ (Zhao et al., 1994) and Cd, Pb and Zn concentrations in plant tissues were determined using AAS.

6.2.3.3 Soil physicochemical parameters

Soil was collected, sieved to <2 mm, and air-dried until constant weight. Subsamples for the determination of soil water content at field capacity were sieved to <4 mm. Soil water content at field capacity was determined by desorption at 10 kPa using a pressure plate apparatus (Soilmoisture Equipment Corp., USA) as described by Richards (1948). Soil pH was measured with a pH-metre in a soil suspension with deionized water (1:2.5, w/v). Total concentrations of Cd, Pb and Zn in soil were determined as described above. For the estimation of metal bioavailability, $CaCl_2$-extractable (0.01 M) Cd, Pb and Zn fractions in soil were obtained following Houba et al. (2000) and then analysed by AAS. Total carbon and total nitrogen content were determined by elementary analysis after dry combustion (LECO TruSpec CHN-S, LECO Corp., USA) following ISO

10694 (1995) and ISO 13878 (1998), respectively. Organic carbon content was measured according to Yeomans and Bremner (1989).

6.2.3.4 Soil microbial parameters

Soil was collected and sieved to <2 mm and stored fresh at 4 ºC until analysis (subsamples for molecular analysis were stored at -20 ºC). A battery of enzyme activities were determined: arylsulphatase, β-glucosidase and acid phosphatase activities were measured according to Dick (1997) and Taylor et al. (2002); urease activity was determined according to Kandeler and Gerber (1988); β-glucosaminidase was determined following Parham and Deng (2000); dehydrogenase activity was measured following ISO 23753-2 (2005); fluorescein diacetate (FDA) hydrolysis activity was determined according to Shaw et al. (2006). Microbial biomass carbon (MBC) was measured by the fumigation-extraction method (Vance et al., 1987). Basal and substrate-induced respiration (SIR) were measured following ISO 16072 (2002) and ISO 17155 (2002), respectively. Soil suppressiveness was evaluated with the agar ring test (Grünwald et al., 1997)) using *Rhizoctonia solanum* as reference organism.

DNA was extracted from soil samples (0.25 g DW soil) using Power Soil™ DNA Isolation Kit (MO BIO Laboratories, USA). Prior to DNA extraction, soil samples were washed twice in 120 mM K_2HPO_4 (pH 8.0) to wash away extracellular DNA (Kowalchuk et al., 1997). Community-level genetic profiles were obtained with ARISA following Cardinale et al. (2004) for bacteria and Ranjard et al. (2000) for fungi. Data were analysed with GeneMarker software (Softgenetics LCC, State College, USA) as described in Welkie et al. (2010).

6.2.4 Field study

An endophyte-assisted phytostabilization field experiment was carried out in May 2014 in the abovementioned mine by inoculating a consortium of five different bacterial endophytes in order to test their plant growth-promoting effect on the native vegetation present in the mine. In particular, a 50 m-long transect was chosen as the experimental area. For a detailed soil physicochemical characterization of this area, see Epelde et al. (2010b). For the consortium, from each of the five plants used here for the isolation of endophytes (see above), one endophyte isolate was chosen, according to its plant growth-promoting characteristics (i.e. within each plant, the isolate showing the best combination of plant-growth-promoting traits). Bacterial endophyte cultures were

grown and prepared as above-stated. The consortium of endophytes was prepared by mixing equal volumes of individual overnight cultures. Prior to the application, the native vegetation present in each plot was cut down to about 2 cm above the soil. Then, the consortium was applied to 15 plots (50 x 50 cm), with 2 m space between plots, along the 50 m-long transect, by means of spraying 0.5 litres of the consortium culture over the vegetation and soil within each plot. Non-inoculated plots (15 plots, 50 x 50 cm) were also included in the experiment as controls, where vegetation was sprayed with sterile LB medium. Treatments (inoculated and non-inoculated) were applied following a randomized design. Two more subsequent inoculations were applied one and two weeks after the first application.

Nine weeks after the first application, plant shoots were cut within each plot. Plant samples were washed thoroughly with deionized water, gently dried with paper towels, separated into excluders (*Festuca rubra*) and accumulators (*Noccaea caerulescens*, *Rumex acetosa*, *Jasione montana* and *Plantago lanceolata*) [see Epelde et al. (2010b) for details], and oven-dried at 70 ºC for 48 h to calculate dry weights. Subsamples were digested and metal (Cd, Pb, Zn) concentrations were determined as described above. Four soil cores (diameter: 2.5 cm; depth: 10 cm) were collected at random within each plot, and mixed together to form a composite sample. Immediately after collection, soil samples were sieved to <2 mm and air-dried until constant weight. Total and $CaCl_2$-extractable metal concentrations in soil were determined as above-described. The following values (mean value ± SD; n = 30) were found for total metal concentrations (mg kg^{-1} DW soil): 11 ± 6, 7,237 ± 1,928 and 30,641 ± 12,053; and $CaCl_2$-extractable metal concentrations (mg kg^{-1} DW soil): 1.0 ± 0.3, 16 ± 5 and 497 ± 138 for Cd, Pb and Zn, respectively.

6.2.5 Statistical analysis

For the growth chamber study, and in order to explore the relationship between experimental factors and soil quality, data on soil metal concentrations and microbial parameters were analysed by means of redundancy analysis (RDA) and variation partitioning analysis using Canoco 5 (ter Braak and Šmilauer, 2012). RDA and variation partitioning were also chosen to investigate the influence of treatments on plant physiological parameters and plant metal concentrations. The effects of experimental factors (*F. rubra* variety, absence or presence of amendment, absence or presence of endophyte inoculation) and their interactions on plant parameters, soil physicochemical

parameters and soil microbial parameters were evaluated by means of three-way ANOVA using IBM SPSS Statistics 19.0. When a significant interaction was found, differences (p<0.05) among levels of factors were tested using the Fisher's Least Significant Difference (LSD) test.

The provision of ecosystem services was calculated from data on soil physicochemical and microbial parameters, based on the index proposed by Epelde et al. (2014b):

$$ES = 10^{\log m + \frac{\sum_{i=1}^{n} |\log n_i - \log m|}{n}}$$

where m is the control value (set to 100%) and n corresponds to the measured values for each parameter as a percentage of the control value. This index is appropriate for those cases where the soil has been intentionally treated to increase the values of some soil parameters (Epelde et al., 2014b), as done in this study. Soil parameters were grouped within ecosystem services as follows: (1) *biodiversity*: richness (S), Shannon's diversity (H') and Pielou's evenness (J') of bacterial and fungal communities from ARISA profiles; (2) *nutrient cycling*: enzyme activities and basal respiration; (3) *carbon storage*: total C content and MBC (MBC accounts for a very small fraction of the total C content in soil; however, soil microorganisms have a key role in the carbon cycle and, to emphasize this point, we have decided to include MBC during the calculation of the *carbon storage* ecosystem service); (4) *water flow regulation*: water content at field capacity*;* (5) *water purification*: pH and $CaCl_2$-extractable metal concentrations; (6) *contamination control*: pH, $CaCl_2$-extractable and total metal concentrations; (7) *pest control*: soil suppressiveness; (8) *fertility maintenance*: total N and organic C content. The arithmetic mean of these eight ecosystem services was calculated as an index of overall soil quality (SQI). Three-way ANOVAs were also calculated for these ecosystem services.

In the field study, when evaluating the effect of the consortium inoculation on plant biomass and plant metal concentrations, one-way ANOVA was conducted for data comparison of excluders and accumulators, separately, using IBM SPSS Statistics 19.0.

6.3 Results

6.3.1 Isolation of bacterial endophytes

Based on their colony morphotype, a total of 78 endophyte strains were isolated from

Table 6.1. Selection of endophytes: plant growth-promoting traits, phenotypic characterization and identification.

		Plant growth-promoting traits					Phenotypic characterization							
Isolate	**Host**	**Organ**	**ACC**	**IAA**	**Sid**	**PO_4**	**Salt**	**Cd**	**Pb**	**Zn**	**NUCS**	**NARS**	**H'**	**Identification**
FS4	*F. rubra*	Stem	3.32 ± 0.34	24.4 ± 0.2	+	+	5	3	6	10	8	2	3	*Pseudomonas* sp
NL2	*N. caerulescens*	Leaf	0.48 ± 0.08	13.8 ± 0.1	-	+	3	0.5	6	5	2	2	1	*Microbacterium* sp.
RR1	*R. acetosa*	Root	0.63 ± 0.10	30.8 ± 0.1	+	+	4	2	8	5	1	5	0	*Pseudomonas* sp.
SR5	*S. atrocinerea*	Root	1.18 ± 0.13	13.8 ± 0.1	+	+	5	2	6	5	1	4	0	*Pseudomonas* sp.
BL4	*B. alba*	Leaf	1.28 ± 0.07	43.7 ± 0.1	+	+	5	3	8	10	1	6	0	*Pseudomonas* sp.

ACC, 1-aminocyclopropane-1-carboxylate deaminase activity (µmol mg^{-1} h^{-1}); IAA, indole-3-acetic acid production (µ$g^{-1}ml^{-1}$); Sid, siderophore production; PO_4, phosphate solubilising activity; Salt, salt tolerance (%); Cd, Maximal Tolerable Concentration (MTC) (mM Cd); Pb, MTC (mM Pb); Zn, MTC (mM Zn); NUCS, number of utilised carbon substrates; NARS, number of antibiotic resistance substrates; H', Shannon´s index

the interior tissues of five plant species collected from the mine: *F. rubra*, *N. caerulescens*, *R. acetosa*, *B. alba* and *S. atrocinerea*. Among these 78 isolates, 31 isolates contained ACC deaminase activity ranging from 0.41 to 3.32 µmol mg^{-1} h^{-1} and thus were subsequently analysed in terms of plant growth-promotion traits, phenotypic characterization and identification (Supplementary Table 6.1). Based on their plant growth-promoting traits, one isolate from each plant species was chosen: one *Microbacterium* sp. strain from *N. caerulescens* and four *Pseudomonas* sp. strains from the other four plant species (Table 6.1).

6.3.2 Growth chamber study

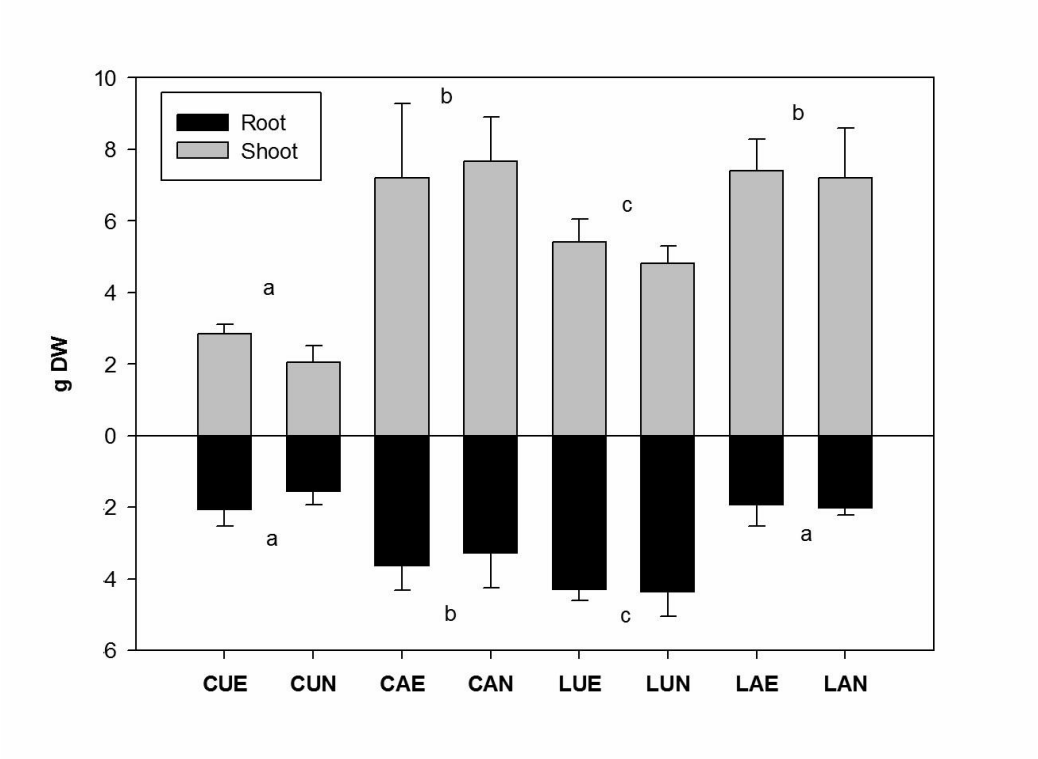


Figure 6.1. Effect of treatments on *F. rubra* shoot and root biomass (g DW). Mean values (n = 3) ± SD. Treatments with different letters are significantly (P<0.05) different according to Fisher's LSD-test. Probability values from three-way ANOVA (ns: non-significant; *, ** and *** represent significances of P<0.05, 0.01 and 0.001, respectively) for shoot and root biomass: plant variety, **, *; amendment, ***, ns; endophyte, ns, ns; plant variety x amendment, **, ***; plant variety x endophyte, ns, ns; amendment x endophyte, ns, ns; plant x amendment x endophyte, ns, ns. CUE: commercial, unamended, inoculated with endophyte; CUN: commercial, unamended, non-inoculated with endophyte; CAE: commercial, amended, inoculated with endophyte; CAN: commercial, amended, non-inoculated with endophyte; LUE: Lanestosa, unamended, inoculated with endophyte; LUN: Lanestosa, unamended, non-inoculated with endophyte; LAE: Lanestosa, amended, inoculated with endophyte; LAN: Lanestosa, amended, non-inoculated with endophyte

According to ANOVA, a significant interaction was found between *F. rubra* variety and amendment for root and shoot biomass (data not shown). Based on this interaction, the significance of the differences among means is shown in Figure 6.1. Values of shoot biomass were significantly higher in amended pots in both commercial and Lanestosa *F. rubra* pots, followed by Lanestosa-unamended pots. Similarly, the application of amendment increased root biomass for the commercial *F. rubra*, but had the opposite effect on Lanestosa *F. rubra*. Endophyte inoculation (*Pseudomonas* sp. FS4) had no effect on plant biomass.

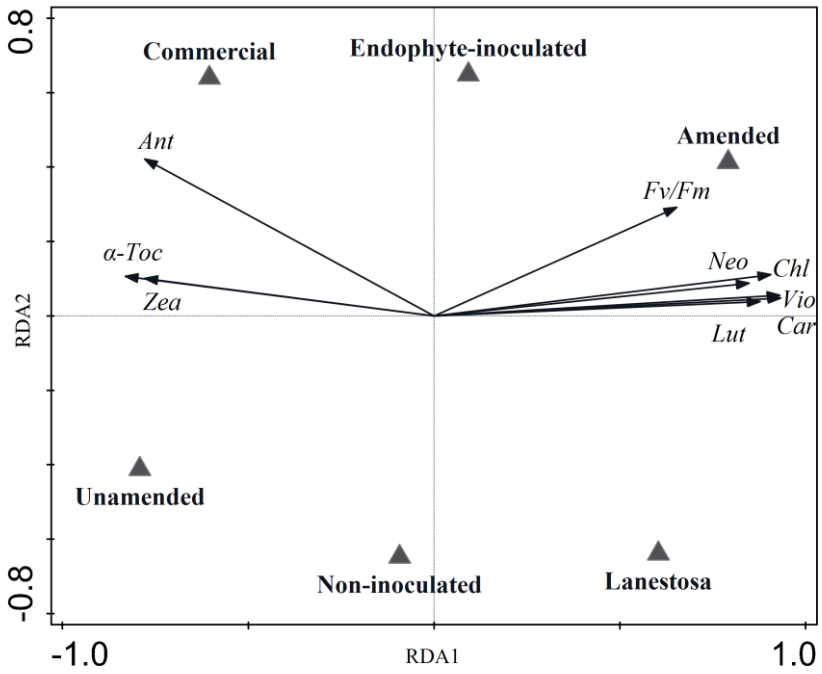


Figure 6.2. Biplot of the redundancy analysis (RDA) from content of lipophilic antioxidants, photoprotective and photosynthetic pigments, and photochemical efficiency; RDA1 and RDA2 account for 71 and 4% of the variance, respectively. Fv/Fm: photochemical efficiency; Vio: violaxanthin; Ant: antheraxanthin; Zea: zeaxanthin; Lut: lutein; Neo: neoxanthin; Car: carotenoids; Chl: chlorophylls a and b; α-Toc: α-tocopherol. Closed symbols represent the different levels of the experimental factors.

Figure 6.2 shows the influence of the experimental factors on plant physiological parameters (F = 19.6, P < 0.01). Some photoprotective pigments [Neo, Vio, Lut, Car], chlorophyll content (Chl) and photochemical efficiency (Fv/Fm) increased towards the positive region of RDA1, which accounted for 71% of the variance, being positively correlated with Lanestosa *F. rubra* and amendment. On the contrary, Ant, Zea and α-Toc contents increased in the commercial *F. rubra* and unamended pots. According to

the variation partitioning analysis, *F. rubra* variety and amendment accounted for 27 and 45% of the explained variation, respectively. Instead, endophyte inoculation explained only 2% of the variation and was not statistically significant. However, a positive influence of endophyte inoculation on some plant physiological parameters was observed in unamended pots: Car and Chl content in endophyte-inoculated Lanestosa *F. rubra* increased 69 and 65%, respectively, compared to non-inoculated plants, while Fv/Fm in endophyte-inoculated commercial *F. rubra* increased 37% (Supplementary Table 6.2).

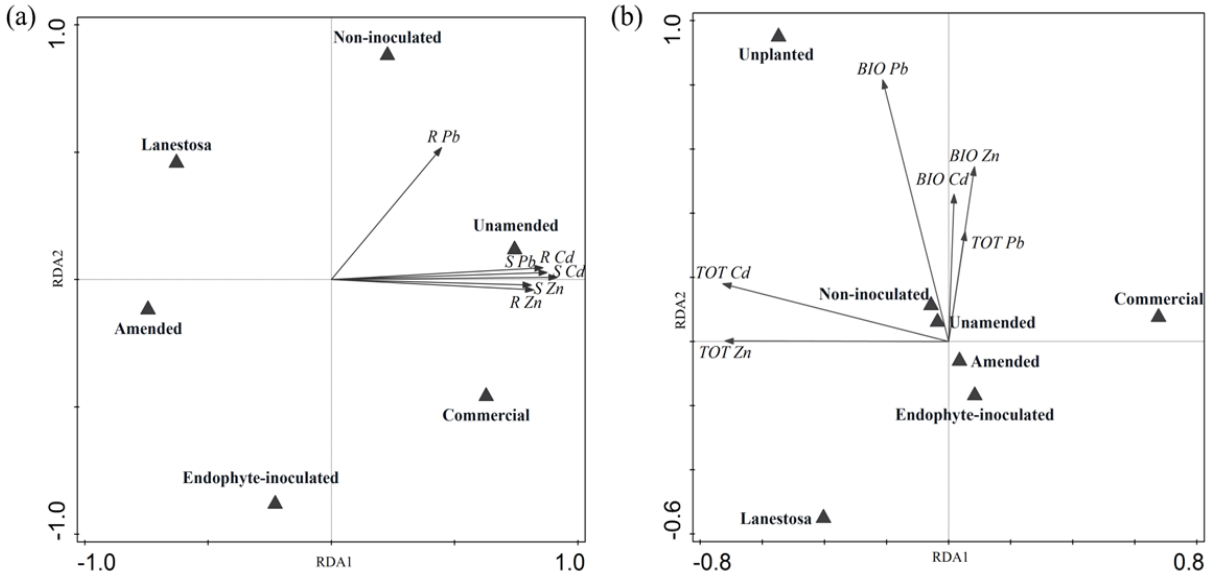


Figure 6.3. (a) Biplot of the redundancy analysis (RDA) from Cd, Pb and Zn concentrations in roots (R) and shoot (S); RDA1 and RDA2 account for 69 and 2% of the total variance, respectively. (b) Biplot of the redundancy analysis (RDA) from Cd, Pb and Zn total (TOT) and $CaCl_2$-extractable (BIO) concentrations; RDA1 and RDA2 account for 50 and 1% of the total variance, respectively. Closed symbols represent the different levels of the experimental factors.

The effects of experimental factors on plant metal concentrations are shown in Figure 6.3a (F = 17.2, P<0.01). Our RDA analysis shows that the commercial *F. rubra* and the absence of amendment resulted in higher metal concentrations in roots and shoots: according to the variation partitioning analysis, *F. rubra* variety and amendment accounted for 28 and 38% of the explained variation, respectively. Regarding endophyte inoculation (6% of explained variation), lower metal concentrations were found in endophyte-inoculated plants, in particular among unamended pots, as confirmed by the interaction obtained between amendment and endophyte inoculation (Supplementary Table 6.3). Values of the translocation factor (i.e., metal concentration in shoots divided

by metal concentration in roots) corroborated the abovementioned effect of *F. rubra* variety and amendment. The lower amount of metal phytoextracted in amended Lanestosa *F. rubra* highlighted the effect of the combination of both factors (Supplementary Table 6.3).

Figure 6.3b (F = 6.3, P<0.01) shows that the growth of commercial *F. rubra* resulted in lower total Cd and Zn concentrations in soil, compared to those found in Lanestosa and unplanted pots. The growth of Lanestosa *F. rubra* decreased Cd and Zn bioavailability in soil, while both the application of the amendment and endophyte inoculation had a smaller influence. This was corroborated by the variation partitioning analysis which showed that *F. rubra* variety explained 49% of the variation, while amendment and endophyte inoculation had no significance. However, values of $CaCl_2$-extractable metal concentrations and relative metal bioavailability (i.e., $CaCl_2$-extractable metal concentration divided by total metal concentration) for Cd, Pb and Zn revealed that the combination of Lanestosa *F. rubra* and amendment application significantly decreased metal bioavailability in soil, as indicated by the interaction found between *F. rubra* variety and amendment (Supplementary Table 6.4). The interaction between amendment and endophyte inoculation corroborated that $CaCl_2$-extractable Cd and Zn concentrations were lower with endophyte inoculation among unamended pots only.

Pertaining to soil microbial parameters, the application of the amendment generally increased the values of the majority of soil microbial parameters. Thus, our RDA analysis of microbial activity parameters (Fig. 6.4a; F = 36.1, P<0.01) showed several enzyme activities (β-glucosidase, urease, phosphatase, β-glucosaminidase) and basal respiration values increasing along the first axis, which accounts for 74% of the total variation, while arylsulphatase activity decreased. According to the variation partitioning analysis, amendment application accounted for 71% of the explained variation, while *F. rubra* variety and endophyte inoculation explained only 8 and 2% of the variation, respectively. Figure 6.4b (F = 44.3, P<0.01) shows a similar correlation between treatments and microbial biomass parameters in which *F. rubra* variety and amendment application explained 8 and 76% of the variation, respectively, and endophyte inoculation had no significance. Notwithstanding, the interaction observed between *F. rubra* variety and amendment application for the majority of measured parameters (Supplementary Table 6.5) indicates that results actually depended on both factors. It is worth mentioning that amendment application decreased the values of

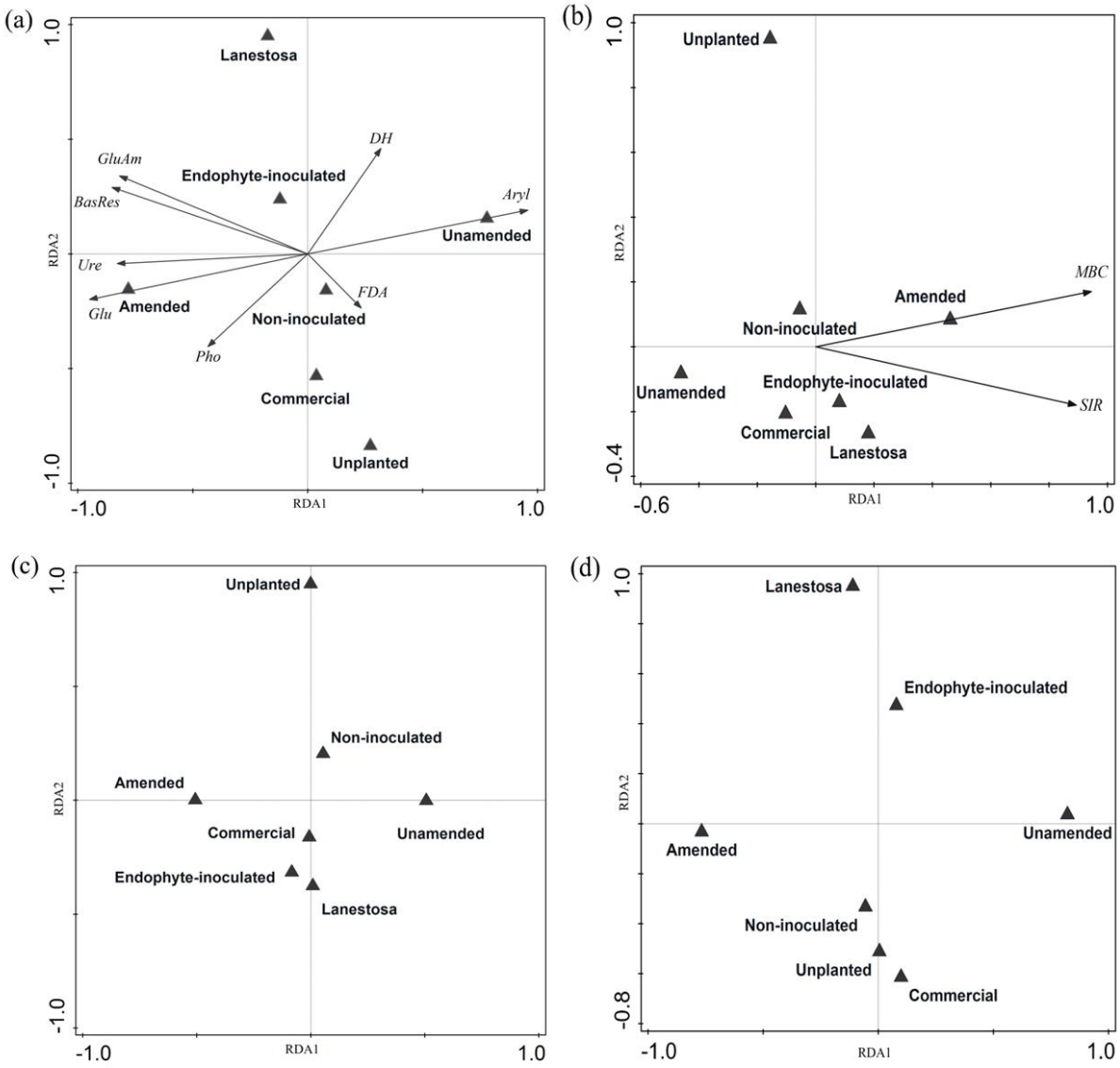


Figure 6.4. Biplots of the redundancy analysis (RDA) performed on: (a) microbial activity parameters; RDA1 and RDA2 account for 74 and 6% of the variance, respectively; (b) microbial biomass parameters; RDA1 and RDA2 account for 84 and 3% of the variance, respectively; (c) bacterial ARISA community profiles; RDA1 and RDA2 account for 43 and 6% of the variance, respectively; (d) fungal ARISA community profiles; RDA1 and RDA2 account for 28 and 6% of the variance, respectively. Pho: phosphatase; Glu: β-glucosidase; GluAm: β-glucosaminidase; Aryl: arylsulphatase; DH: dehydrogenase; FDA: fluorescein diacetate hydrolysis; Ure: urease; MBC: microbial biomass carbon; BasRes: basal respiration; SIR: substrate-induced respiration. Closed symbols represent the different levels of the experimental factors.

dehydrogenase activity in pots with Lanestosa *F. rubra*, while no variation was observed with the commercial *F. rubra*. On the other hand, endophyte inoculation positively influenced FDA and urease activity in pots with commercial *F. rubra* and dehydrogenase activity in pots with Lanestosa *F. rubra*, but always in the absence of

amendment. Finally, concerning soil microbial diversity parameters, RDAs of community-level ARISA profiles showed that amendment application was the factor with the strongest effect on bacterial (Fig. 6.4c; F = 6.8, P<0.05) and fungal communities (Fig. 6.4d; F = 3.8, P<0.01), with 43 and 28% of explained variation, respectively, followed by *F. rubra* variety explaining 6 and 6% of the variation, respectively.

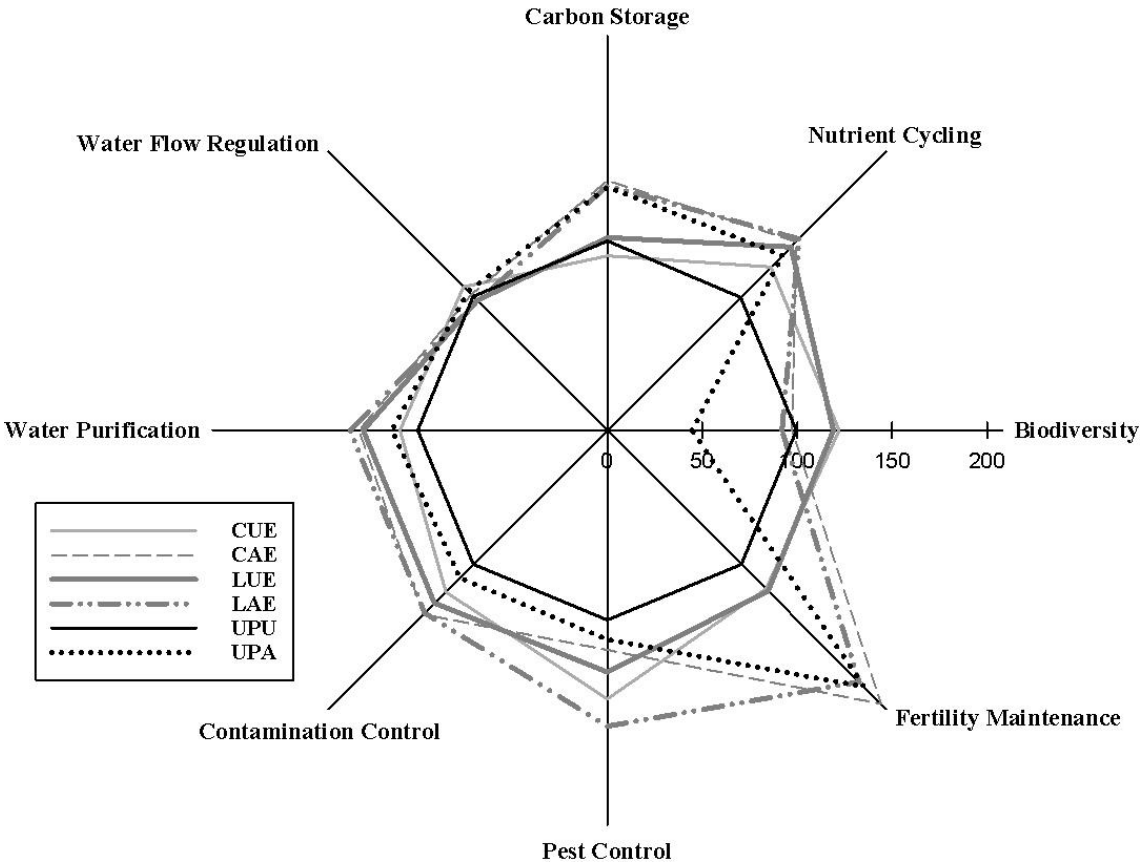


Figure 6.5. Sunray plot of ecosystem services. A value of 100 corresponds to the mean value obtained for each ecosystem service in the unplanted, unamended (UPU) control treatment. CUE: commercial, unamended, inoculated with endophyte; CAE: commercial, amended, inoculated with endophyte; LUE: Lanestosa, unamended, inoculated with endophyte; LAE: Lanestosa, amended, inoculated with endophyte; UPU: unplanted, unamended; UPA: unplanted, amended.

A visual illustration of the overall effect of treatments on ecosystem services is presented in Figure 6.5. In order to facilitate interpretation, only endophyte-inoculated and unplanted treatments are shown. This figure shows the positive effect of Lanestosa *F. rubra* and amendment on *nutrient cycling*, *water purification* and *contamination control*, compared to the unplanted, unamended control. *Carbon storage* and *fertility*

maintenance increased particularly in amended pots, while *water flow regulation* seemed to be influenced solely by *F. rubra* variety. *Biodiversity* was positively influenced by the growth of both *F. rubra* varieties, as opposed to unplanted pots, but was negatively affected by amendment application. Higher values of *biodiversity*, *nutrient cycling* and *pest control*, particularly among unamended pots, were caused by endophyte inoculation.

Finally, according to the Soil Quality Index (SQI, Supplementary Table 6.7), the presence of both Lanestosa *F. rubra* and the amendment had a significant positive effect on soil quality, while the influence of endophyte inoculation was observed mostly among unamended pots.

6.3.3 Field experiment

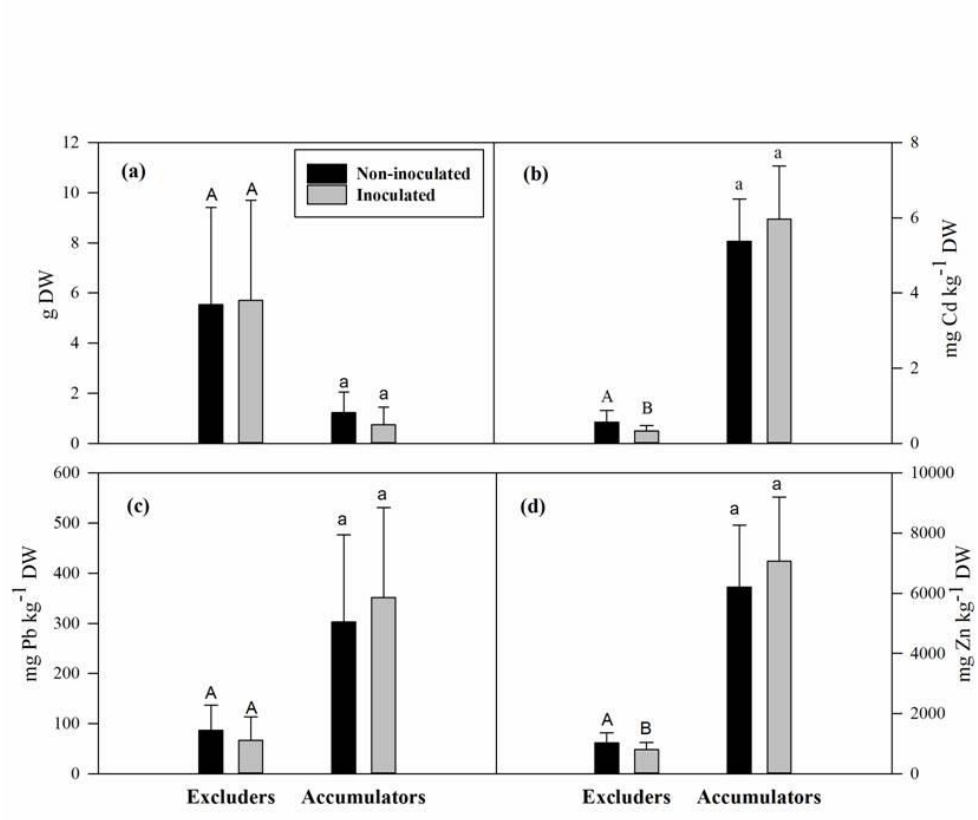


Figure 6.6. Plant biomass (a; g DW) and plant concentrations (mg kg^{-1}) of Cd (b), Pb (c) and Zn (d) for excluder and accumulator plants in the endophyte-assisted phytostabilization field experiment. Mean values (n = 15) ± SD. Bars labelled with different letters are significantly (P<0.05) different according to Fisher's LSD-test (in capital letters for excluders and lowercase letters for accumulators).

The field inoculation of the consortium had no effect on plant biomass of excluders or accumulators (Fig. 6.6a). Regarding shoot metal concentration (Fig. 6.6b, c

and d), although no significant effects were obtained, increasing values of Cd, Pb and Zn were observed among accumulator plants inoculated with the consortium, compared to control. On the other hand, shoot Cd and Zn concentrations in excluder plants were significantly lower when inoculated with the consortium

6.4 Discussion

6.4.1 Isolation of bacterial endophytes

Mine tailings frequently have a poor physical structure, high levels of available metals and low nutrient concentrations, resulting in a strong selective pressure on local biodiversity. As a consequence, mine tailings usually present a unique assemblage of plant species and associated microbial communities with great potential for application for phytomanagement (Barrutia et al., 2011). Here, endophytic bacteria were isolated from five (pseudo)metallophyte species present in an abandoned mine, including herbaceous plants (*F. rubra, N. caerulescens, R. acetosa*) and tree species (*B. alba, S. atrocinerea*).

From a total of 78 isolates, 31 (39%) had ACC deaminase activity, which is reported to be the major mechanism used by bacteria to promote plant growth by lowering plant ethylene levels (Glick, 2014), and were then characterized for other plant growth-promoting traits. Based on their plant growth-promoting traits, five isolates (one from each plant species) were selected as candidates for endophyte-assisted phytostabilization (Table 6.1). Four of the five strains were assigned to the genus *Pseudomonas*, which is among the most common genera of cultivable endophytes and includes numerous plant growth-promoting bacterial species (Barzanti et al., 2007; Rashid et al., 2012; Liu et al., 2014). These four *Pseudomonas* sp. strains displayed medium levels of ACC deaminase activity and IAA production, together with a high tolerance to metals. They also exhibited other plant growth-promoting traits, such as siderophore production and phosphate solubilising activity, that can alleviate metal toxicity and enhance plant growth by increasing the supply of iron to the plant (Burd et al., 2000) and increasing available phosphate (Park et al., 2011), respectively, thereby making these strains promising candidates for endophyte-assisted phytoremediation.

6.4.2 Growth chamber study

The application of cow slurry had a pronounced effect on *F. rubra* growth, increasing both shoot and root biomass. This positive effect of the organic amendment masked the

influence of the other treatments. If, for instance, we examine the results of unamended pots only, we observe that Lanestosa *F. rubra*, compared to the commercial variety, shows an inherent advantage to overcome the problems associated with growing in our mine soil, as reflected by the fact that, in the absence of amendment, the shoots of commercial *F. rubra* were very chlorotic and its shoot biomass was 48% lower than that of Lanestosa *F. rubra* (Fig. 6.1). The root:shoot biomass ratio of Lanestosa *F. rubra* was greater in unamended than amended pots. In the absence of amendment (and the nutrients provided therein), plants must put more energy into establishing a root system with a large surface area to aid in nutrient uptake (Grandlic et al., 2008). Ideally, plants for phytostabilization should develop an extensive root system and produce a large amount of biomass in the presence of high concentrations of metals, while keeping translocation to aerial parts as small as possible to prevent the entry of these contaminants into the food web (Gómez-Sagasti et al., 2012). In this respect, Lanestosa *F. rubra* in unamended pots showed larger root biomass than commercial *F. rubra*.

On the other hand, values of plant physiological parameters in unamended pots revealed a positive effect due to endophyte inoculation: higher values of carotenoids, chlorophylls, and Fv/Fm were observed in endophyte inoculated plants of both *F. rubra* varieties (Supplementary Table 6.2), suggesting an amelioration of the plant physiological status. This could be due to ACC deaminase activity leading to a reduction of stress-induced ethylene levels in *F. rubra* plants.

The concentrations of Cd, Pb and Zn in the shoots of commercial *F. rubra* reached 8.9, 351 and 4,156 mg kg^{-1}, respectively, which were higher than values found in Lanestosa *F. rubra*. Indeed, Lanestosa *F. rubra*, despite its larger shoot biomass, presented a lower amount of metal phytoextracted, suggesting that commercial *F. rubra* plants were not able to control the uptake and translocation of metals from the roots, being severely affected by metal toxicity. Moreover, the growth of Lanestosa *F. rubra* significantly decreased soil metal bioavailability, indicating that phytostabilization with native plants (tolerant to metals as well as to the other adverse environmental factors prevailing in these mining sites) is a better option to stabilize metal contaminants.

In addition to the incorporation of nutrients to the soil, the input of organic matter from cow slurry has been reported to contribute to metal immobilization and to reduce metal bioavailability (Galende et al., 2014a). In our study, amendment application reduced soil metal bioavailability, leading to lower metal concentrations in plants, particularly in shoots (Supplementary Table 6.3). Indeed, a decrease in the

translocation factor values in amended pots was observed, which could be due to metal dilution in plant tissues as a consequence of plant growth. This indicated an effect of the amendment not just on plant metal uptake, but also on the resulting translocation to aerial parts.

On the other hand, the decomposition of organic matter with time after amendment application may release metals initially bound to the amendment (Martínez et al., 2003). In this respect, endophyte inoculation decreased soil metal concentrations without the aid of the amendment. Endophytes able to produce metal chelators, such as siderophores, can reduce metal toxicity for their host plants and affect the translocation of metal from roots to shoots (Mastretta et al., 2006; Weyens et al., 2009). Besides, Park et al. (2011) reported that the release of phosphate to solution by phosphate solubilising bacteria may facilitate metal immobilization. This, together with the improved physiological status of endophyte-inoculated plants, indicates that endophytes isolated from native metal-tolerant plants and with plant growth-promoting traits may be capable of conferring metal resistance and eliciting physiological changes that modulate the growth and development of plants, providing them with fitness to counter the problems associated with mine soil such as nutrient deficiency and metal toxicity.

As already stated, apart from dealing with contaminants, the main aim of any remediation process must be to restore soil quality. Here, we have described how the application of cow slurry and the use of Lanestosa *F. rubra* successfully decreased soil metal bioavailability. But for a more accurate estimation of the reduction in metal toxicity, and resulting improvement of soil quality, soil microbial parameters have great potential as biological indicators of soil quality. Indeed, microbial communities play a key role in many soil processes and the delivery of soil ecosystem services (Jeffery et al., 2010). Particularly, soil enzymes have been recommended as standard biochemical indicators to assess the quality of metal contaminated soils (Gómez-Sagasti et al., 2012).

Here, amendment application and, to a lesser extent, the growth of Lanestosa *F. rubra* resulted in an increase in the values of soil microbial parameters. Organic amendments can reduce metal bioavailability, as well as to increase soil microbial activity due to the input of easily biodegradable organic matter (Bolan et al., 2011). Plant growth has also been reported to enhance microbial properties of mine soils (Epelde et al., 2010b). However, in opposition to other enzyme activities, arylsulphatase activity decreased with amendment application. Enzyme activities have been found to respond differently to the same metal contamination, depending on the specific enzyme

activity, metal and soil property (Burges et al., 2015). Similarly, a certain phytoremediation treatment could either increase or decrease the values of soil enzyme activities. Amendment application had a different effect on dehydrogenase activity only in pots with Lanestosa *F. rubra*, resulting in lower values. These results go in accordance with the lower root biomass found in Lanestosa *F. rubra* in the same pots, suggesting a positive correlation between dehydrogenase activity and the root system developed by the plants. Plant biomass has been reported to be positively correlated with soil microbial parameters, mainly due to the development of rhizobacteria induced by the release of specific sugars and amino acids into the rhizosphere by the plant (Epelde et al., 2010b).

Pertaining to endophyte inoculation, although it did not seem to have any significant effect on the majority of soil microbial parameters, higher values of FDA, dehydrogenase and urease activities were found in the absence of amendment, suggesting again that the effect of endophyte inoculation was disguised by the effect of the amendment. This effect was observed regardless of the *F. rubra* variety, suggesting no specificity of the endophytes with plant variety. Bacteria isolated from plants have shown to promote growth of other plant species, in many cases belonging to distinct botanical groups (Sessitsch et al., 2013).

In order to allow an easier interpretation of soil quality results, we based the concept of soil quality on soil ecosystem services, by grouping soil parameters (especially, soil microbial parameters) within a set of ecosystem services. The use of Lanestosa *F. rubra* seemed to preferentially improve *carbon storage*, as reflected in MBC values, as well as *biodiversi*ty and *nutrient cycling*; besides, it reduced soil metal contamination, as reflected by a decrease in the *contamination control* values. Again, this positive effect confirms the superior phytostabilization efficiency of Lanestosa *F. rubra*, over the commercial variety, attributed to improvements in soil quality due to a better developed root system.

The application of cow slurry resulted in an increase in *nutrient cycling*, *carbon storage* and *fertility maintenance*, which are ecosystem services that rely on soil microbial activity, primary productivity and, in general, functionality of the soil ecosystem; on the other hand, it also increased *water purification* and *contamination control*, which are related to the potential of the soil ecosystem to counter metal toxicity. This effect is presumably due to the reduction in soil metal bioavailability and the input of easily biodegradable organic matter resulting from the application of cow

slurry (Alvarenga et al., 2009a, 2009b). However, despite the expected stimulation of soil microbial activity and biomass, the application of cow slurry induced an adverse impact on bacterial and fungal diversity resulting in lower *biodiversity* values. The greater amount of organic matter carried by the amendment may have promoted the intense reproduction of certain microbial groups, to the detriment of others, resulting in a decrease in microbial diversity (Tian et al., 2015).

The inoculation of the endophyte also improved some ecosystem services (i.e., *biodiversity*, *nutrient cycling* and *pest control*), but this effect was only observed in the absence of amendment. *Pest control* seemed to be influenced solely by endophyte inoculation, conferring the soil with the capacity to prevent disease development despite the presence of a pathogen.

The SQI, calculated from the values of the ecosystem services, can be interpreted as an integrative measurement of the effectiveness of the endophyte-assisted aided phytostabilization, providing valuable information on the overall status of the soil ecosystem. Nonetheless, it must be interpreted with caution as it is only a simplified reflection of the extremely complex soil ecosystem and, inevitably, entails a loss of information about the response of each specific parameter considered during the calculation of the SQI (Burges et al., 2015).

Interestingly, the conclusions obtained from the evaluation of ecosystem services resemble those obtained separately from soil parameters. Notwithstanding, the advantage of this approach relies on the fact that these higher, more-general categories are easier to interpret (i.e., the more the better) and, above all, should be *a priori* much less affected by changes in techniques, methods, equipment, interests, etc. (Epelde et al., 2014a). In any case, when possible, it is desirable to assess soil quality at both levels as they may provide complementary information.

6.4.3 Field experiment

Many studies have been successfully conducted on the use of plants assisted by metal tolerant plant growth-promoting bacteria in metal phytoremediation (Grandlic et al., 2008; Zhang et al., 2011; Liu et al., 2014). Nonetheless, the majority of these studies have been done in pots under controlled conditions, and not many results from field experiments are available. Therefore, we decided to further investigate the potential benefit of inoculating a consortium of five selected endophytes in an endophyte-assisted phytostabilization field experiment.

The inoculation of the consortium had no effect on the growth of the native metallophyte vegetation present in the mine. On the other hand, lower concentration of Cd and Zn were observed in consortium-inoculated excluder plants, suggesting that the consortium could have a positive effect on excluders by reducing plant metal uptake.

6.5 Conclusions

Although the application of cow slurry is a suitable option for phytostabilization (here, it significantly improved soil quality and plant colonization), it can entail a number of environmental problems such as reduction of microbial biodiversity. In this respect, in the absence of amendment, the native variety of *F. rubra* successfully decreased soil metal bioavailability and improved the soil microbial community in terms of activity, biomass and diversity. On the other hand, endophyte-inoculation reduced plant metal uptake in the absence of amendment, and enhanced plants´ physiological status, thus providing plants with fitness to counter the problems associated with mine soil, resulting in soil quality improvement. However, further studies are needed to determine the ecological fitness of endophytes before they are used in field-scale applications. Our results highlight the potential of the biodiversity (plants and microorganisms) from metalliferous environments for phytomanagement. Finally, the assessment of soil quality through the grouping of soil parameters in ecosystem services has demonstrated to be an appropriate tool to estimate the effectiveness of phytoremediation.

Supplementary Table 6.1. Endophytes: plant growth-promoting traits, phenotypic characterization and identification.

			Plant growth-promoting traits				Phenotypic characterization							
Strain	**Host**	**Organ**	**ACC**	**IAA**	**Sid**	**PO_4**	**Salt**	**Cd**	**Pb**	**Zn**	**NUCS**	**NARS**	**H'**	**Identification**
FR1	*F. rubra*	Root	0.97 ± 0.09	14.2 ± 0.1	-	-	2	1	4	5	18	2	4	*Microbacterium* sp.
FR9	*F. rubra*	Root	0.92 ± 0.06	5.3 ± 0.1	-	+	2	3	8	5	19	2	4	*Plantibacter* sp.
FR10	*F. rubra*	Root	0.49 ± 0.02	11.2 ± 0.1	-	-	3	0.5	4	5	11	2	3	*Microbacterium* sp.
FR12	*F. rubra*	Root	0.66 ± 0.03	-	-	+	5	2	6	15	7	2	3	*Rhodococcus* sp.
FS1	*F. rubra*	Root	0.72 ± 0.05	7.1 ± 0.1	+	+	4	2	6	10	18	2	4	*Pseudomonas* sp
FS3	*F. rubra*	Stem	0.99 ± 0.02	40.7 ± 0.2	-	-	3	2	4	5	6	1	3	*Agreia* sp.
FS4	*F. rubra*	Stem	3.32 ± 0.34	24.4 ± 0.2	+	+	5	3	6	10	8	2	3	*Pseudomonas* sp.
NR1	*N. caerulescens*	Root	3.28 ± 0.40	13.5 ± 0.1	-	-	0.5	5	6	5	24	1	4	*Variovorax* sp.
NR2	*N. caerulescens*	Root	0.51 ± 0.06	-	-	-	2	2	4	10	12	2	4	*Microbacterium* sp.
NS3	*N. caerulescens*	Stem	1.31 ± 0.10	-	-	-	2	1	4	5	13	2	4	*Microbacterium* sp.
NS9	*N. caerulescens*	Stem	0.41 ± 0.07	-	-	-	7	0.5	4	1	22	0	2	*Brevibacterium* sp.
NL1	*N. caerulescens*	Leaf	0.74 ± 0.23	58.9 ± 1.3	-	-	5	1	4	2.5	1	1	0	*Micrococcus* sp.
NL2	*N. caerulescens*	Leaf	0.48 ± 0.08	13.8 ± 0.1	-	+	3	0.5	6	5	2	2	1	*Microbacterium* sp.
NL4	*N. caerulescens*	Leaf	0.67 ± 0.03	-	-	-	7	0.5	6	0.5	17	0	3	*Brevibacterium* sp.
RR1	*R. acetosa*	Root	0.63 ± 0.10	30.8 ± 0.1	+	+	4	2	8	5	1	5	0	*Pseudomonas* sp.
RR2	*R. acetosa*	Root	0.66 ± 0.09	33.0 ± 0.1	+	+	4	2	6	5	1	5	0	*Pseudomonas* sp.
RR3	*R. acetosa*	Root	1.04 ± 0.03	35.1 ± 0.5	-	-	3	0.5	2	5	42	2	5	*Microbacterium* sp.
RS1	*R. acetosa*	Stem	1.10 ± 0.21	83.5 ± 8.0	-	+	2	1	6	5	29	2	5	*Plantibacter* sp.
RL2	*R. acetosa*	Leaf	0.89 ± 0.11	84.7 ± 3.7	-	-	2	1	6	5	25	2	5	*Microbacterium* sp.
SR3	*S. atrocinerea*	Root	1.14 ± 0.06	29.5 ± 1.3	-	-	7	<0.5	4	0.5	7	3	2	*Microbacterium* sp.
SR5	*S. atrocinerea*	Root	1.18 ± 0.13	13.8 ± 0.1	+	+	5	2	6	5	1	4	0	*Pseudomonas* sp.
SS2	*S. atrocinerea*	Stem	1.65 ± 0.10	28.6 ± 2.1	-	-	5	<0.5	4	1	7	4	3	*Micrococcus* sp.
SS4	*S. atrocinerea*	Stem	0.53 ± 0.08	29.1 ± 0.4	-	-	3	1	4	0.5	12	2	3	*Staphylococcus* sp.
SL1	*S. atrocinerea*	Leaf	1.54 ± 0.11	-	-	-	7	<0.5	4	1	10	3	3	*Brevibacterium* sp.
BR1	*B. alba*	Root	1.06 ± 0.13	30.1 ± 0.4	-	-	2	1	4	5	10	1	3	*Microbacterium* sp.
BR4	*B. alba*	Root	0.92 ± 0.05	-	-	-	7	0.5	6	1	14	3	4	*Brevibacterium* sp.

Strain	Host	Organ	Plant growth-promoting traits				Phenotypic characterization							Identification
			ACC	IAA	Sid	PO_4	Salt	Cd	Pb	Zn	NUCS	NARS	H'	
BS1	*B. alba*	Stem	1.05 ± 0.09	41.0 ± 0.4	-	-	7	<0.5	4	0.5	3	2	2	*Micrococcus* sp.
BL3	*B. alba*	Leaf	1.19 ± 0.16	6.3 ± 0.1	+	+	4	1	6	2.5	16	6	4	*Enterobacteriaceae*
BL4	*B. alba*	Leaf	1.28 ± 0.07	43.7 ± 0.1	+	+	5	3	8	10	1	6	0	*Pseudomonas* sp.
BL5	*B. alba*	Leaf	0.74 ± 0.03	8.2 ± 0.2	+	+	5	2	6	5	1	5	0	*Pseudomonas* sp.
BL7	*B. alba*	Leaf	1.01 ± 0.03	18.1 ± 0.3	+	+	4	2	4	5	40	2	5	*Microbacterium* sp.

ACC, 1-aminocyclopropane-1-carboxylate deaminase activity (µmol mg^{-1} h^{-1}); IAA, indole-3-acetic acid production ($\mu g^{-1} ml^{-1}$); Sid, siderophore production; PO_4, phosphate solubilising activity; Salt, salt tolerance (%); Cd, Maximal Tolerable Concentration (MTC) (mM Cd); Pb, MTC (mM Pb); Zn, MTC (mM Zn); NUCS, number of utilised carbon substrates; NARS, number of antibiotic resistance substrates; H', Shannon´s index.

Supplementary Table 6.2. Effect of treatments on plant physiological parameters (Chl, VAZ, A+Z/VAZ, Car and α-Toc in nmol g^{-1} FW). Mean values (n = 3) ± SD. Probability values from three-way ANOVA (ns: non-significant) for the effects of *F. rubra* variety, amendment and endophyte and their interactions are shown below. CUE: commercial, unamended, inoculated with endophyte; CUN: commercial, unamended, non-inoculated with endophyte; CAE: commercial, amended, inoculated with endophyte; CAN: commercial, amended, non-inoculated with endophyte; LUE: Lanestosa, unamended, inoculated with endophyte; LUN: Lanestosa, unamended, non-inoculated with endophyte; LAE: Lanestosa, amended, inoculated with endophyte; LAN: Lanestosa, amended, non-inoculated with endophyte.

	Fv/Fm	**Chl**	**VAZ**	**A+Z/VAZ**	**Car**	**α-Toc**
CUE	0.70 ± 0.07	598 ± 31	55 ± 3	0.38 ± 0.05	224 ± 11	224 ± 32
CUN	0.51 ± 0.02	495 ± 46	50 ± 6	0.45 ± 0.04	190 ± 20	211 ± 3
CAE	0.78 ± 0.02	2827 ± 85	150 ± 5	0.06 ± 0.01	894 ± 36	44 ± 11
CAN	0.77 ± 0.02	2971 ± 403	166 ± 22	0.04 ± 0.01	976 ± 191	50 ± 10
LUE	0.73 ± 0.03	2965 ± 532	160 ± 18	0.04 ± 0.01	952 ± 137	74 ± 15
LUN	0.69 ± 0.11	1795 ± 128	92 ± 2	0.04 ± 0.01	562 ± 46	53 ± 5
LAE	0.76 ± 0.01	3679 ± 179	157 ± 4	0.03 ± 0.01	1145 ± 29	44 ± 7
LAN	0.76 ± 0.02	3535 ± 82	165 ± 5	0.04 ± 0.00	1121 ± 42	43 ± 6
Plant variety (P)	<0.05	<0.001	<0.001	<0.001	<0.001	<0.001
Amendment (A)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Endophyte (E)	<0.01	<0.01	<0.05	ns	<0.05	ns
P x A	<0.01	<0.001	<0.001	<0.001	<0.001	<0.001
P x E	ns	<0.01	<0.01	ns	<0.01	ns
A x E	<0.05	<0.01	<0.001	<0.05	<0.01	ns
P x A x E	ns	ns	<0.01	ns	ns	ns

Fv/Fm: photochemical efficiency; Chl: chlorophylls a and b; V: violaxanthin; A: antheraxanthin; Z: zeaxanthin; Car: carotenoids; α-Toc: α-tocopherol.

Supplementary Table 6.3. Effect of treatments on *F. rubra* root and shoot metal concentrations (mg kg^{-1} DW) and translocation factors. Mean values (n = 3) ± SD. Probability values from three-way ANOVA (ns: non-significant) for the effects of *F. rubra* variety, amendment and endophyte and their interactions are shown below. CUE: commercial, unamended, inoculated with endophyte; CUN: commercial, unamended, non-inoculated with endophyte; CAE: commercial, amended, inoculated with endophyte; CAN: commercial, amended, non-inoculated with endophyte; LUE: Lanestosa, unamended, inoculated with endophyte; LUN: Lanestosa, unamended, non-inoculated with endophyte; LAE: Lanestosa, amended, inoculated with endophyte; LAN: Lanestosa, amended, non-inoculated with endophyte.

	Root Concentration			**Shoot Concentration**			**Translocation Factor**			**Metal phytoextracted**		
	Cd	**Pb**	**Zn**	**Cd**	**Pb**	**Zn**	**Cd**	**Pb**	**Zn**	**Cd**	**Pb**	**Zn**
CUE	20.9 ± 1.1	2577 ± 234	9211 ± 868	5.4 ± 1.7	245 ± 63	3873 ± 117	0.26 ± 0.09	0.10 ± 0.03	0.42 ± 0.03	0.014 ± 0.004	0.61 ± 0.05	10.0 ± 2.3
CUN	26.4 ± 2.0	3210 ± 531	9821 ± 714	8.9 ± 1.8	351 ± 28	4156 ± 446	0.34 ± 0.07	0.11 ± 0.02	0.42 ± 0.02	0.019 ± 0.008	0.72 ± 0.21	8.6 ± 2.7
CAE	14.9 ± 1.3	2274 ± 45	7180 ± 354	3.2 ± 0.6	114 ± 26	1612 ± 378	0.22 ± 0.06	0.05 ± 0.01	0.22 ± 0.05	0.023 ± 0.006	0.80 ± 0.02	11.1 ± 1.3
CAN	15.4 ± 1.1	3834 ± 180	8406 ± 1149	2.8 ± 0.3	107 ± 4	1598 ± 340	0.18 ± 0.03	0.03 ± 0.00	0.19 ± 0.03	0.021 ± 0.001	0.82 ± 0.13	11.9 ± 0.9
LUE	14.2 ± 1.4	3240 ± 309	6242 ± 801	2.9 ± 0.5	104 ± 15	1906 ± 116	0.20 ± 0.03	0.03 ± 0.01	0.31 ± 0.03	0.017 ± 0.002	0.63 ± 0.29	11.2 ± 3.6
LUN	17.2 ± 0.5	3877 ± 575	9142 ± 287	4.4 ± 1.1	176 ± 20	2782 ± 502	0.25 ± 0.06	0.05 ± 0.01	0.30 ± 0.07	0.021 ± 0.005	0.85 ± 0.13	13.4 ± 3.6
LAE	11.8 ± 1.0	2140 ± 140	6159 ± 1434	1.2 ± 0.1	84 ± 16	595 ± 37	0.10 ± 0.02	0.04 ± 0.01	0.10 ± 0.02	0.008 ± 0.003	0.53 ± 0.04	3.9 ± 1.1
LAN	11.7 ± 1.3	2422 ± 294	4445 ± 399	1.2 ± 0.3	81 ± 23	700 ± 306	0.10 ± 0.02	0.03 ± 0.01	0.15 ± 0.05	0.001 ± 0.003	0.67 ± 0.03	5.7 ± 3.0
Plant (P)	<0.001	ns	<0.001	<0.001	<0.001	<0.001	<0.05	<0.001	<0.001	<0.05	ns	ns
Amendment (A)	<0.001	<0.01	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	ns	ns	<0.05
Endophyte (E)	<0.01	<0.001	<0.05	<0.05	<0.01	<0.05	ns	ns	ns	ns	ns	ns
P x A	<0.01	<0.001	ns	ns	<0.001	<0.05	ns	<0.001	<0.001	<0.001	ns	<0.001
P x E	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
A x E	<0.01	ns	<0.05	<0.01	<0.01	<0.05	ns	ns	ns	ns	ns	ns
P x A x E	ns	ns	<0.01	ns	ns	ns	ns	ns	ns	ns	ns	ns

Supplementary Table 6.4. Effect of treatments on total and $CaCl_2$-extractable metal concentrations (mg kg^{-1} DW) and relative metal bioavailability ([metal]bioavailable/[metal]total). Mean values (n = 3) ± SD. Probability values from three-way ANOVA (ns: non-significant) for the effects of *F. rubra* variety, amendment and endophyte and their interactions are shown below. CUE: commercial, unamended, inoculated with endophyte; CUN: commercial, unamended, non-inoculated with endophyte; CAE: commercial, amended, inoculated with endophyte; CAN: commercial, amended, non-inoculated with endophyte; LUE: Lanestosa, unamended, inoculated with endophyte; LUN: Lanestosa, unamended, non-inoculated with endophyte; LAE: Lanestosa, amended, inoculated with endophyte; LAN: Lanestosa, amended, non-inoculated with endophyte; UPU: unplanted, unamended; UPA: unplanted, amended.

	Total			**$CaCl_2$-extractable**			**Relative bioavailability**		
	Cd	**Pb**	**Zn**	**Cd**	**Pb**	**Zn**	**Cd**	**Pb**	**Zn**
CUE	7.9 ± 0.3	4346 ± 160	10955 ± 1436	1.67 ± 0.04	8.8 ± 0.4	577 ± 6	21.3 ± 1.4	0.20 ± 0.01	5.3 ± 0.6
CUN	8.7 ± 0.7	4203 ± 599	10323 ± 1385	1.72 ± 0.04	8.8 ± 0.4	584 ± 22	19.9 ± 1.4	0.21 ± 0.02	5.7 ± 0.8
CAE	9.0 ± 0.5	4015 ± 684	11700 ± 595	1.24 ± 0.09	6.8 ± 1.1	412 ± 34	13.8 ± 1.3	0.17 ± 0.05	3.5 ± 0.3
CAN	8.9 ± 0.8	4379 ± 44	12250 ± 715	1.18 ± 0.02	7.0 ± 0.4	400 ± 11	13.3 ± 1.4	0.16 ± 0.01	3.3 ± 0.2
LUE	9.9 ± 0.1	4323 ± 480	14663 ± 1196	1.42 ± 0.15	6.1 ± 0.9	448 ± 44	14.5 ± 1.4	0.14 ± 0.02	3.1 ± 0.2
LUN	10.2 ± 0.8	3633 ± 244	15310 ± 1756	1.57 ± 0.04	7.6 ± 0.4	505 ± 7	15.5 ± 1.5	0.21 ± 0.01	3.3 ± 0.4
LAE	9.8 ± 1.1	3942 ± 488	13719 ± 1475	1.19 ± 0.16	6.3 ± 0.7	387 ± 43	12.2 ± 1.3	0.16 ± 0.03	2.8 ± 0.3
LAN	12.5 ± 1.1	4262 ± 36	12119 ± 275	1.11 ± 0.02	7.3 ± 0.2	384 ± 11	9.0 ± 0.8	0.17 ± 0.00	3.2 ± 0.1
UPU	11.8 ± 0.5	4430 ± 88	14021 ± 1231	1.75 ± 0.03	13.0 ± 1.1	578 ± 22	14.8 ± 0.8	0.29 ± 0.03	4.1 ± 0.2
UPA	12.2 ± 0.4	4387 ± 390	14611 ± 1086	1.37 ± 0.02	10.0 ± 0.7	486 ± 44	11.2 ± 0.5	0.23 ± 0.01	3.3 ± 0.3
Plant variety (P)	<0.001	ns	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Amendment (A)	<0.001	ns	ns	<0.001	<0.001	<0.001	<0.001	<0.01	<0.001
Endophyte (E)	<0.05	ns	ns	ns	ns	ns	ns	ns	ns
P x A	ns	ns	<0.01	<0.05	<0.001	<0.001	<0.01	<0.05	<0.001
P x E	ns	ns	ns	ns	<0.05	ns	ns	ns	ns
A x E	ns	ns	ns	<0.01	ns	<0.05	ns	ns	ns
P x A x E	ns	ns	ns	ns	ns	ns	ns	ns	ns

Supplementary Table 6.5. Effect of treatments on soil microbial parameters. Mean values (n = 3) ± SD. Probability values from three-way ANOVA (ns: non-significant) for the effects of *F. rubra* variety, amendment and endophyte and their interactions are shown below. CUE: commercial, unamended, inoculated with endophyte; CUN: commercial, unamended, non-inoculated with endophyte; CAE: commercial, amended, inoculated with endophyte; CAN: commercial, amended, non-inoculated with endophyte; LUE: Lanestosa, unamended, inoculated with endophyte; LUN: Lanestosa, unamended, non-inoculated with endophyte; LAE: Lanestosa, amended, inoculated with endophyte; LAN: Lanestosa, amended, non-inoculated with endophyte; UPU: unplanted, unamended; UPA: unplanted, amended.

	Pho	Glu	GluAm	Aryl	DH	FDA	Ure	MBC	BasRes	SIR	S Bact	H' Bact	J' Bact	S Fungi	H' Fungi	J' Fungi
CUE	245 ± 12	84 ± 4	31 ± 1	76 ± 5	8.8 ± 0.9	300 ± 4	8.9 ± 0.3	124 ± 7	1.5 ± 0.3	6.7 ± 1.9	51 ± 4	5.0 ± 0.1	0.88 ± 0.02	14 ± 3	2.8 ± 0.1	0.75 ± 0.09
CUN	249 ± 3	90 ± 2	32 ± 2	78 ± 4	8.8 ± 0.4	263 ± 11	4.9 ± 0.6	113 ± 8	1.2 ± 0.9	6.5 ± 0.1	47 ± 2	5.0 ± 0.1	0.90 ± 0.01	9 ± 1	2.1 ± 0.2	0.67 ± 0.08
CAE	291 ± 10	137 ± 3	60 ± 6	28 ± 3	9.3 ± 0.3	219 ± 4	11.0 ± 1.5	219 ± 5	3.6 ± 0.6	31.0 ± 7.7	52 ± 7	4.8 ± 0.4	0.84 ± 0.05	6 ± 3	2.1 ± 0.3	0.74 ± 0.03
CAN	273 ± 14	133 ± 5	55 ± 4	25 ± 4	9.2 ± 0.1	208 ± 15	10.6 ± 0.6	195 ± 9	3.4 ± 0.4	31.4 ± 1.7	48 ±3	4.3 ± 0.4	0.77 ± 0.06	3 ± 1	1.6 ± 0.0	0.90 ± 0.08
LUE	261 ± 15	87 ± 5	57 ± 4	80 ± 0	11.1 ± 0.5	256 ± 58	6.5 ± 0.6	150 ± 15	2.3 ± 0.2	16.6 ± 2.2	49 ±7	5.0 ± 0.0	0.89 ± 0.04	14 ± 4	2.7 ± 0.5	0.73 ± 0.05
LUN	261 ± 15	86 ± 1	52 ± 4	79 ± 5	10.1 ± 0.5	185 ± 20	6.0 ± 1.1	156 ± 9	1.7 ± 0.4	15.5 ± 2.5	43 ± 7	4.9 ± 0.2	0.90 ± 0.02	6 ± 2	1.8 ± 0.4	0.61 ± 0.08
LAE	274 ± 4	126 ± 4	70 ± 1	25 ± 1	8.6 ± 0.6	239 ± 42	11.5 ± 0.1	226 ± 20	4.4 ± 1.8	35.7 ± 6.5	50 ±7	4.4 ± 0.3	0.78 ± 0.03	4 ± 1	1.9 ± 0.0	0.90 ± 0.06
LAN	260 ± 8	124 ± 11	78 ± 9	27 ± 1	7.5 ± 0.6	193 ± 22	10.0 ± 0.3	212 ± 4	4.4 ± 0.4	31.9 ± 2.7	32 ± 3	4.2 ± 0.2	0.85 ± 0.03	6 ± 2	2.2 ± 0.5	0.88 ± 0.08
UPU	281 ± 5	79 ± 0	32 ± 3	69 ± 5	8.2 ± 0.2	208 ± 6	3.5 ± 1.3	141 ± 8	1.2 ± 0.2	10.2 ± 0.7	45 ± 5	4.8 ± 0.2	0.87 ± 0.02	11 ± 3	1.8 ± 0.5	0.55 ± 0.09
UPA	306 ± 12	126 ± 12	72 ± 14	32 ± 5	6.7 ± 0.4	253 ± 5	11.4 ± 1.1	209 ± 3	1.8 ± 0.3	16.0 ± 0.8	40 ± 5	4.1 ± 0.2	0.77 ± 0.06	3 ± 1	0.3 ± 0.0	0.18 ± 0.04
Plant variety (P)	<0.001	<0.05	<0.001	ns	<0.001	<0.05	ns	<0.001	<0.01	<0.001	<0.05	ns	ns	ns	<0.001	<0.001
Amendment (A)	<0.001	<0.001	<0.001	<0.001	<0.001	ns	<0.001	<0.001	<0.001	<0.001	ns	<0.001	<0.001	<0.001	<0.001	ns
Endophyte (E)	ns	ns	ns	ns	<0.05	<0.01	<0.001	ns	ns	ns	<0.01	ns	ns	<0.01	<0.01	ns
P x A	<0.01	ns	<0.05	<0.01	<0.001	<0.01	<0.01	<0.05	<0.05	<0.01	ns	ns	ns	ns	<0.001	<0.001
P x E	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	<0.05	ns	ns	ns
A x E	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	<0.05	<0.05	<0.05
P x A x E	ns	ns	ns	ns	ns	ns	<0.01	ns	ns	ns	ns	ns	ns	ns	ns	ns

Supplementary Table 6.6. Effect of treatments on other soil physicochemical and biological parameters. Mean values (n = 3) ± SD. Probability values from three-way ANOVA (ns: non-significant) for the effects of *F. rubra* variety, amendment and endophyte and their interactions are shown below. CUE: commercial, unamended, inoculated with endophyte; CUN: commercial, unamended, non-inoculated with endophyte; CAE: commercial, amended, inoculated with endophyte; CAN: commercial, amended, non-inoculated with endophyte; LUE: Lanestosa, unamended, inoculated with endophyte; LUN: Lanestosa, unamended, non-inoculated with endophyte; LAE: Lanestosa, amended, inoculated with endophyte; LAN: Lanestosa, amended, non-inoculated with endophyte; UPU: unplanted, unamended; UPA: unplanted, amended.

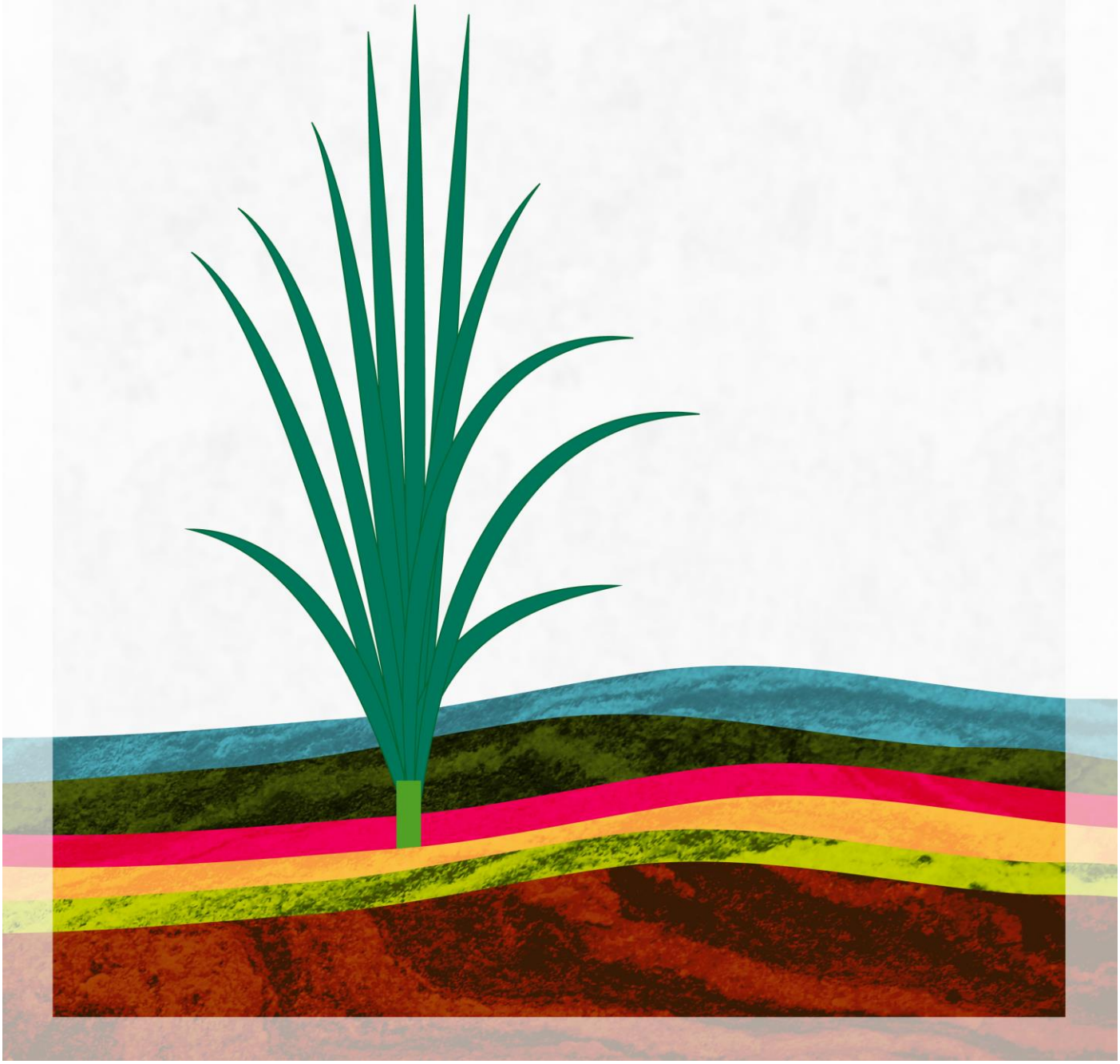
	pH	Total C	Total N	SWC	Organic C	Suppress
CUE	6.6 ± 0.0	3.1 ± 0.2	0.28 ± 0.02	32.9 ± 1.2	65 ± 4	0.32 ± 0.03
CUN	6.5 ± 0.0	3.0 ± 0.2	0.27 ± 0.01	32.7 ± 1.1	59 ± 12	0.42 ± 0.07
CAE	6.5 ± 0.2	3.6 ± 0.2	0.34 ± 0.02	31.1 ± 1.0	162 ± 12	0.39 ± 0.07
CAN	6.5 ± 0.1	3.6 ± 0.4	0.31 ± 0.04	31.3 ± 1.5	163 ± 4	0.43 ± 0.06
LUE	6.6 ± 0.1	3.1 ± 0.4	0.28 ± 0.02	29.8 ± 0.8	68 ± 0	0.36 ± 0.07
LUN	6.5 ± 0.0	3.0 ± 0.4	0.27 ± 0.02	30.5 ± 1.4	62 ± 7	0.43 ± 0.05
LAE	6.5 ± 0.2	3.4 ± 0.3	0.32 ± 0.03	29.6 ± 1.4	146 ± 11	0.29 ± 0.01
LAN	6.6 ± 0.1	3.7 ± 0.2	0.33 ± 0.02	30.4 ± 0.9	172 ± 15	0.38 ± 0.05
UPU	6.5 ± 0.0	3.2 ± 0.2	0.28 ± 0.03	30.7 ± 0.9	37 ± 4	0.45 ± 0.02
UPA	6.2 ± 0.1	3.6 ± 0.1	0.33 ± 0.03	31.8 ± 0.7	147 ± 4	0.36 ± 0.04
Plant variety (P)	<0.01	ns	ns	<0.01	<0.001	ns
Amendment (A)	<0.01	<0.001	<0.001	ns	<0.001	ns
Endophyte (E)	ns	ns	ns	ns	ns	<0.01
P x A	ns	ns	ns	ns	ns	ns
P x E	ns	ns	ns	ns	ns	ns
A x E	ns	ns	ns	ns	<0.05	ns
P x A x E	ns	ns	ns	ns	ns	ns

Total C: total carbon content (%); Total N: total nitrogen content (%); SWC: soil water content at field capacity (%); Organic C: organic carbon content (mg C kg^{-1}); Suppress: soil suppressiveness.

Supplementary Table 6.7. Effect of treatments on soil ecosystem services and the soil quality index (SQI). Values are expressed as a percentage of the unplanted, unamended control (UPU). Mean values (n = 3) ± SD. Probability values from three-way ANOVA (ns: non-significant) for the effects of F. rubra variety, amendment and endophyte and their interactions are shown below. CUE: commercial, unamended, inoculated with endophyte; CUN: commercial, unamended, non-inoculated with endophyte; CAE: commercial, amended, inoculated with endophyte; CAN: commercial, amended, non-inoculated with endophyte; LUE: Lanestosa, unamended, inoculated with endophyte; LUN: Lanestosa, unamended, non-inoculated with endophyte; LAE: Lanestosa, amended, inoculated with endophyte; LAN: Lanestosa, amended, non-inoculated with endophyte; UPU: unplanted, unamended; UPA: unplanted, amended.

	Biodiversity	**Nutrient cycling**	**Carbon storage**	**Water flow regulation**	**Water purification**	**Contamination control**	**Pest control**	**Fertility maintenance**	**SQI**
CUE	122 ± 4	122 ± 5	92 ± 3	107 ± 4	109 ± 0	120 ± 3	142 ± 14	118 ± 3	117 ± 2
CUN	105 ± 5	110 ± 4	87 ± 6	106 ± 4	108 ± 1	120 ± 3	107 ± 19	109 ± 11	107 ± 2
CAE	97 ± 11	141 ± 3	132 ± 3	101 ± 3	131 ± 9	137 ± 4	116 ± 24	204 ± 14	132 ± 4
CAN	85 ± 4	133 ± 7	125 ± 8	102 ± 5	132 ± 3	135 ± 3	104 ± 14	198 ± 14	127 ± 3
LUE	119 ± 12	137 ± 2	102 ± 10	97 ± 3	129 ± 9	129 ± 8	128 ± 27	120 ± 5	120 ± 3
LUN	89 ± 12	122 ± 6	105 ± 5	99 ± 5	117 ± 2	119 ± 3	104 ± 11	113 ± 9	108 ± 3
LAE	92 ± 2	143 ± 7	129 ± 8	97 ± 5	136 ± 10	136 ± 9	156 ± 8	188 ± 7	135 ± 3
LAN	93 ± 10	137 ± 6	132 ± 5	99 ± 3	134 ± 1	130 ± 1	117 ± 15	208 ± 3	131 ± 2
UPU	100 ± 13	99 ± 5	100 ± 2	100 ± 3	100 ± 1	100 ± 1	100 ± 4	100 ± 7	100 ± 3
UPA	45 ± 2	130 ± 6	129 ± 2	104 ± 2	113 ± 3	110 ± 6	110 ± 10	191 ± 8	117 ± 3
Plant variety (P)	<0.001	<0.001	<0.001	<0.01	<0.001	<0.001	ns	ns	<0.001
Amendment (A)	<0.001	<0.001	<0.001	ns	<0.001	<0.001	ns	<0.001	<0.001
Endophyte (E)	<0.01	<0.001	ns	ns	ns	ns	<0.01	ns	<0.001
P x A	<0.001	<0.05	<0.05	ns	<0.05	<0.05	ns	ns	ns
P x E	ns	ns	ns	ns	ns	ns	ns	ns	ns
A x E	<0.05	ns	ns	ns	ns	ns	ns	ns	<0.05
P x A x E	ns	ns	ns	ns	ns	ns	ns	ns	ns

07 | MICROBIAL PROPERTIES AND ATTRIBUTES OF ECOLOGICAL RELEVANCE FOR SOIL QUALITY MONITORING DURING A CHEMICAL STABILIZATION FIELD STUDY



7. MICROBIAL PROPERTIES AND ATTRIBUTES OF ECOLOGICAL RELEVANCE FOR SOIL QUALITY MONITORING DURING A CHEMICAL STABILIZATION FIELD STUDY

Epelde L, Burges A, Mijangos I, Garbisu C, 2014. Microbial properties and attributes of ecological relevance for soil quality monitoring during a chemical stabilization field study. ***Applied Soil Ecology*** *75: 1-12.*

Abstract

Chemical stabilization is a soil remediation technique based on the incorporation of organic and/or inorganic amendments to metal contaminated soil in order to decrease metal bioavailability and improve soil quality. Consequently, the establishment of follow-up monitoring programs is essential to ensure the long-term effectiveness of chemical stabilization in terms of both metal bioavailability reduction and soil quality improvement. In this study, three doses (20, 40 and 80 t ha^{-1}) of a lime-treated sewage sludge, that meets legal standards regarding metal contents, were added to a metalliferous mine soil and a variety of physicochemical and microbial indicators of soil quality were measured over time (immediately before treatment application and one and six months after such application). Soil $CaCl_2$-extractable and plant metal concentrations were also measured. We carried out a complementary interpretation of soil microbial properties through their grouping within a set of ecosystem attributes of ecological relevance: vigour, organization, stability, suppressiveness and redundancy. Sewage sludge addition led to an increase in soil pH, but this beneficial effect was transient. The addition of sewage sludge had a more pronounced effect on parameters used here to estimate soil vigour (dehydrogenase activity, basal and substrate-induced respiration). On the contrary, the addition of sewage sludge did not significantly alter the composition of soil microbial communities, as reflected by PCR-DGGE data. Chemical stabilization was only partly successful: it did improve soil quality but the expected reduction in soil metal bioavailability (as reflected by the values of $CaCl_2$-extractable metal concentration) was clearly observed only for Cd (not for Pb or Zn); however, SL addition led to a significant reduction in shoot metal concentration for the three metals under study. The assessment of soil quality at the attribute level has proven useful for the interpretation of the effect of chemical stabilization on soil functioning.

7.1 Introduction

Mine soils are frequently unfavourable environments for living organisms due to high levels of bioavailable metals, acidity, lack of organic matter (OM) and associated nutrients, and poor substrate structure (Tordoff et al., 2000; Wong, 2003). The incorporation of organic and/or inorganic amendments to metal contaminated soils, followed by a chemical stabilization period, aims to reduce metal bioavailability and improve soil quality through the increase of soil pH, OM content, nutrient content and water-holding capacity (Alvarenga et al., 2009a; 2009b). Then, the establishment of follow-up monitoring programs is essential to ensure the long-term effectiveness of chemical stabilization.

The main goal of any soil remediation technology is to remove the contaminant(s) from the site or to render them harmless but restoring the soil quality, *i.e*. the capacity of soil to perform its functions (Doran and Parkin, 1994; Karlen et al., 2003). Soil microbial properties are well-known valuable indicators of soil quality; in particular, microbial properties have often been used to assess the recovery of soil quality during phytoremediation of metal contaminated soils (Epelde et al., 2008a; 2009a; Kumpiene et al., 2009; Epelde et al., 2010a). Soil microorganisms have key functions in many vital soil processes, such as OM decomposition and nutrient cycling, and are responsible, in a great extent, for the functioning of soil ecosystems (Nielsen and Winding, 2002; Anderson, 2003; Schloter et al., 2003; Lin et al., 2004).

Soil microbial properties, together with soil physical and chemical parameters, are being increasingly used as bioindicators of soil quality (Karlen et al., 1994; Bastida et al., 2006; Puglisi et al., 2006). With the objective of facilitating the interpretation of soil microbial properties in terms of soil quality through the utilization of less context-dependent (more universal indicators), Garbisu et al. (2011) recently proposed to link the concept of soil quality to that of ecosystem health through grouping of soil microbial properties within a set of ecosystem attributes of ecological relevance, such as *vigour* (a measure of the activity, metabolism or primary productivity of a system), *organization* (which may be assessed in terms of the diversity of components and their degree of mutual dependence), *stability* (a system´s ability to maintain its structure and pattern of behaviour in the presence of stress), *suppressiveness* (the capacity of a given soil to maintain disease severity or incidence at a low level, despite the presence of a pathogen, a susceptible host plant, and climatic conditions favourable for disease development) and *redundancy* (the number of, for instance, species per functional group). These five attributes overlap with each other to a certain extent, but are at

the same time complementary (that is why, when possible, all five attributes should be measured).

The main aim of this work was to validate this approach in a chemical stabilization field study. To this aim, the abovementioned five attributes were quantified in a field experiment carried out in a metalliferous mine soil heavily contaminated with cadmium (Cd), lead (Pb) and zinc (Zn), where increasing doses of a lime-treated sewage sludge were applied. The evolution over time of a variety of soil physicochemical and microbial properties was monitored. We hypothesized that grouping soil microbial properties within ecosystem attributes of ecological relevance could facilitate the interpretation of the effect of chemical stabilization on soil functioning.

7.2 Materials and methods

7.2.1 Site description

The studied area is located in an abandoned Pb-Zn mine (Coto Txomin) in the Western Biscay district, Basque-Cantabrian Basin, northern Spain (43º13´ N, 3º26´ W), within the temperate Atlantic Region of the Iberian Peninsula. The climate is temperate and wet, with no dry season or extreme seasonal changes. Mean annual rainfall in the nearest town (Lanestosa) is about 1,400 mm y^{-1}. Mean annual temperature can vary from 11 to 15 ºC, with a mean value of 18 ºC in July and 5.5 ºC in January. For a more detailed description of the experimental site, see Barrutia et al. (2011).

Within the mine, an experimental area of approximately 10 m^2 was chosen for this study, owing to its flat surface, lack of vegetation and uniform soil physicochemical properties. The soil has the following physicochemical properties: 14.3 mg Cd kg^{-1} dry weight (DW) soil, 13,683 mg Pb kg^{-1} DW soil, 26,810 mg Zn kg^{-1} DW soil, sandy loam texture, an OM content of 4.2%, a pH of 6.7, and an ammonium content of 0.13 mg N-NH_4^+ kg^{-1} DW soil. Total concentration of metals was determined using an Atomic Absorption Spectrometer (Spectra AA-250 plus, Varian, Australia) following aqua regia digestion (McGrath and Cunliffe, 1985).

7.2.2 Experimental setup

Different doses of a lime-treated sewage sludge (20, 40 and 80 t sewage sludge ha^{-1}) were applied to 1 m^2 size plots (n = 3) within the 10 m^2 experimental area, following a randomized block design with a 1 m space between plots. Non-amended plots (n = 3) were included in the experiment as controls. The physicochemical properties of the sewage sludge were the

following: 25.4% dry matter, pH = 8.1, 64.4% OM, 1.38% total nitrogen (on a fresh weight basis), and 2.4, 45, 2310, 347, 68, 1.02 and 138 mg kg^{-1} DW of cadmium, lead, zinc, copper, nickel, mercury and chromium, respectively. Regarding its metal content, the sewage sludge meets the requirements of the Spanish Royal Decree 1310/1990, which regulates the use of sludge from sewage treatment plants in the agricultural sector. Microbiological tests were also performed to look for potential pathogens in the sewage sludge: *Salmonella* sp. and *Ascaris* sp. were absent, and the number of *Escherichia coli* cells was below the threshold established by the Spanish Royal Decree 824/2005 for fertilizers. In any case, the sewage sludge was treated with lime (6% CaO) in order to minimize its bacterial load and, thus, reduce the risk of incorporating potential pathogens into the environment: prior to its incorporation to the soil, the sewage sludge was grounded with a plant debris brush cutter; afterwards, 6% CaO was spread over it and then the mixture was thoroughly homogenized in a mixer. Soil was sampled (upper 0-10 cm) in all the experimental plots (within each plot, a composite soil sample from six randomly selected places was obtained) immediately before the application of lime-treated sewage sludge (SL; initial sampling), and one and six months after such application (these sampling times correspond to February, March and August 2012, respectively). In the last sampling (August), the natural vegetation present in an area of 0.5 m^2, randomly selected within each plot, was cut down to about 1 cm above the soil for metal analysis.

7.2.3 Soil physicochemical parameters

For the analysis of physicochemical parameters, soils were sieved to <2 mm and air-dried until constant weight. For the estimation of metal bioavailability, $CaCl_2$-extractable (0.01 M) Cd, Pb and Zn fractions were obtained following Houba et al. (2000) and then analyzed using an Atomic Absorption Spectrometer. Soil pH was measured with a pH-meter in a soil suspension with deionized water (1:2.5 w:v). Water-soluble organic carbon (WSOC) was extracted and measured according to Epelde et al. (2010b). Ammonium ($N\text{-}NH_4^+$) was extracted using 1 M KCl and then determined following Nelson (1983).

7.2.4 Plant parameters

In the last sampling, harvested plants were washed thoroughly with deionized water and gently dried with paper towels. Fresh weights were recorded and, subsequently, shoots were oven-dried at 70 ºC for 48 h to calculate dry weights. Subsamples (0.2 g) of dried shoot tissue were digested with a mixture of $HNO_3/HClO_4$ (Zhao et al., 1994) and, finally, Cd, Pb and Zn

were determined using an Atomic Absorption Spectrometer (Spectra AA-250 plus, Varian, Australia).

7.2.5 Soil microbial parameters

For microbial parameters, soils were sieved to <2 mm and stored fresh at 4 ºC for a maximum of two months until analysis (subsamples for molecular analysis were stored at -20 ºC). Except for the soil stability test (see below), three biological replicates were used in every analysis. Dehydrogenase activity was determined as described in Epelde et al. (2009a). Basal and substrate-induced respiration were measured following ISO 16072 Norm (2002) and ISO 17155 Norm (2002), respectively. Microbial biomass carbon was determined following Vance et al. (1987). ATP content was measured according to Webster et al. (1984) and Ciardi and Nannipieri (1990): ATP was extracted by ultrasonication of 0.5 g of soil in 7.5 mL of an acid medium (20 mL of 3.33 M phosphoric acid, 20 mL of dimethyl sulfoxide, 20 mL of 10 M urea, 20 mL of 100 mM EDTA, 4 mL of 18.75 mM adenosine, 5 mL of 10% benzalkonium chloride and 11 mL of distilled water), followed by centrifugation at 14,000 rpm for 1 min. Subsequently, 200 μL of the supernatant were neutralized to pH 7.8 ± 0.2 by the addition of 0.3 M Trizma (for each set of analyses, the volume of Trizma required to neutralize the supernatant was previously calculated; it was approximately 5.8 times the volume of the supernatant), followed again by centrifugation at 14,000 rpm for 1 min, to clean the supernatant. For the determination of ATP, standard additions and a commercially available kit, based on the reaction catalyzed by the luciferase enzyme (A22066, Molecular Probes, Paisley), were used. Community-level physiological profiles (CLPPs) were determined with Biolog Ecoplates™ (Hayward; Insam, 1997) following Epelde et al. (2008a). Average well color development (AWCD) and Shannon´s diversity index (H') were calculated after 50 h of incubation, which corresponded to the time of maximal microbial growth in the Biolog EcoPlates™. Soil vigour was calculated from the values of the abovementioned microbial parameters (dehydrogenase activity, basal and substrate-induced respiration, microbial biomass C, ATP content and AWCD; see below).

In order to assess the stability of soil microbial communities against environmental disturbances, a soil stability test against heat stress was carried out. Disturbance and community stability are necessarily related, as stability is defined as a community´s response to disturbance (Shade et al., 2012). For this purpose, two replicates (50 g DW) of each soil sample were preconditioned for one week in plastic pots at 20 ºC in the dark and 60% water-holding capacity. Then, one of the replicates was subjected to 42 ºC for 24 h (Wertz et al.,

2007); the other replicate was left as control (its humidity was kept constant at 60% water-holding capacity throughout the 15 weeks that the test lasted). After 0 and 15 weeks of incubation at 20 ºC in the dark, dehydrogenase activity and nitrification potential rate (ISO 15685, 2004) were determined in all soil samples. Stability is comprised of resistance (degree to which a community is insensitive to a disturbance) and resilience (rate at which a community returns to a pre-disturbance condition) (Shade et al., 2012); in consequence, from the values of dehydrogenase activity and nitrification potential rate, resistance (RS) and resilience (RL) indices were calculated according to Orwin and Wardle (2004):

$$RS(t_0) = 1 - \frac{2|D_0|}{(C_0 + |D_0|)} \qquad RL(t_x) = \frac{2|D_0|}{(|D_0| + |D_x|)} - 1$$

where D_0 is the difference between control (C_0) and disturbed at 0 weeks, while D_x is the difference between control and disturbed at 15 weeks of incubation.

Soil suppressiveness was determined with the agar ring test following Grünwald et al. (1997), as described in Núñez-Zofío et al. (2012), using the fungus *Neurospora crassa* as reference organism. After 72 h of incubation, the radial growth of the fungal colony was measured in two perpendicular directions and averaged. The relative reduction in *N. crassa* growth was calculated as follows: relative growth = radial growth (cm) on non-autoclaved soil / radial growth (cm) on autoclaved soil.

For molecular analyses, DNA was extracted from soil samples (0.25 g DW soil) using Power Soil™ DNA Isolation Kit (MO BIO Laboratories, Carlsbad) according to the manufacturer's specifications. Prior to DNA extraction, soil samples were washed twice in 120 mM K_2HPO_4 (pH 8.0) to wash away extracellular DNA (Kowalchuk et al., 1997). For the estimation of microbial biomass (18S rDNA gene fragments for total fungi, 16S rDNA gene fragments for total bacteria and total archaea), real-time PCR measurements of gene copy abundance were carried out using the primers and PCR conditions shown in Table 7.1. Each 25 μL reaction contained 2.5 μL of template, 12.5 μL of SYBR Premix Ex Taq (Takara Bio Inc., Seta), 0.25 μL of each primer (at a concentration of 20, 30 and 50 μM for fungi, bacteria and archaea, respectively), 1.25 μL bovine serum albumin (40 mg mL^{-1}), 0.5 μL of ROX dye and 7.75 μL of sterile Milli-Q water. Standards were made from plasmids containing the target sequence (Dhanasekaran et al., 2010).

Table 7.1. Primers and PCR conditions.

Primers	PCR conditions	References
Q-PCR for total fungi: Fung5F and FF390R	95 °C for 30 s, 94 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min (40 cycles); 95 °C for 15 s, 60 °C for 1 min, 95 °C for 30 s for the melt curve, with a final extension of 60 °C for 15 s	Lueders et al. (2004a,b)
Q-PCR for total bacteria: Ba519F and Ba907R	95 °C for 30 s, 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min (40 cycles); 95 °C for 15 s, 60 °C for 1 min, 95 °C for 30 s for the melt curve, with a final extension of 60 °C for 15 s	Lueders et al. (2004a,b)
Q-PCR for total archaea: A751F and UA1406R	95 °C for 30 s, 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min (40 cycles); 95 °C for 15 s, 60 °C for 1 min, 95 °C for 30 s for the melt curve, with a final extension of 60 °C for 15 s	Baker et al. (2003)
PCR-DGGE for fungi: FR1-GC and FF390	94 °C for 30 s, 94 °C for 1 min, 50 °C for 45 s, 72 °C for 1 min (30 cycles); a final extension at 72 °C for 5 min	Vainio and Hantula (2000)
PCR-DGGE for bacteria: 968F-GC and 1378R	94 °C for 30 s, 92 °C for 30 s, 55 °C for 1 min, 68 °C for 45 s (+1 s $cycle^{-1}$; 35 cycles); a final extension at 68 °C for 5 min	Heuer et al. (1997)
PCR-DGGE for archaea: PRARCH112F and PREA1100E; PARCH340F-GC and PARCH519R	95 °C for 30 s, 94 °C for 30 s, 51 °C for 30 s, 72 °C for 1 min (35 cycles); a final extension at 72 °C for 10 min, 95 °C for 30 s, 94 °C for 30 s, 72 °C for 30 s, 72 °C for 1 min (-0.5 °C $cycle^{-1}$; 20 cycles); 94 °C for 30 s, 62 °C for 30 s, 72 °C for 1 min (20 cycles); a final extension at 72 °C for 10 min	Hoj et al. (2005); Ovreås et al. (1997)
PCR-DGGE for denitrifying bacteria: CD3AF and R3CD-GC	94 °C for 2 min, 94 °C for 30 s, 57 °C for 1 min, 72 °C for 1 min (35 cycles); a final extension at 72 °C for 10 min	Throbäck et al. (2004)
PCR-DGGE for chitin-degrading bacteria: GA1F and GA1R; GASQF-GC and GASQR	94 °C for 30 s, 94 °C for 1 min, 60 °C for 10 s, 72 °C for 1 min (35 cycles), a final extension at 72 °C for 10 min	Williamson et al. (2000)

PCR-DGGE analyses were performed on soil samples using the primers and PCR conditions shown in Table 7.1. For bacterial communities, the PCR mix was as follows: 25 µL volumes containing 1 µL of template, 12.5 µL of 2X Premix Ex Taq™, 1.5 µL of each primer (10 µM) and 8.5 µL of sterile Milli-Q water. Amplifications were carried out on an iCycler Thermal Cycler (Bio-Rad, Hercules). For the DGGE analysis, a D-Code Universal Mutation Detection System (Bio-Rad, Hercules) was used. The denaturating gradient was from 35 to 60% (100% denaturant is defined as 7 M urea and 40% v/v formamide) and 6% acrylamide. Gradient gels were topped with 5 mL of acrylamide containing no denaturant. DGGE was performed using 20 µL of the PCR product in 1 × TAE buffer at 60 °C. Electrophoresis was performed at 170 V for 10 min followed by 90 V for 15 h. Gels were stained with GelRedTM Nucleic Acid Gel Stain (Biotium, Hayward) following manufacturer's instructions and the bands visualized under UV light in a G:BOX (Syngene, Cambridge). Banding patterns were analyzed using Phoretix 1D (TotalLab, Newcastle) Program. For archaeal communities, PCRs were carried out with PRARCH112F and PREA1100E primers, followed by a second round of amplification with 1 µL of the former PCR product with PARCH340F-GC and PARCH519R primers. The PCR mix was as follows: 25 µL volumes containing 2 µL of template (1 µL in the second round), 12.5 µL of 2X Premix Ex Taq™, 1.25 µL of each primer (10 µM) and 8 µL of sterile Milli-Q water (9 µL in the second round). The denaturating gradient was from 40 to 70% and 8% acrylamide. For denitrifying bacteria (nitrite reductase gene), the PCR mix was as follows: 25 µL volumes containing 2 µL of template, 12.5 µL of 2X Premix Ex Taq™, 2.5 µL of each primer (10 µM) and 5.50 µL of sterile Milli-Q water. The denaturating gradient was from 75 to 82% and 6% acrylamide. PCR mixes, denaturing gradients and electrophoresis conditions for fungal communities and chitin-degrading bacteria were as indicated in Epelde et al. (2012a). Soil organization was calculated from the values of the Shannon´s diversity index (from Biolog Ecoplates™) and from real-time PCR and PCR-DGGE data for fungi, bacteria and archaea (see below). Instead, soil redundancy was calculated from PCR-DGGE data for chitin-degrading and denitrifying bacteria.

The assignment of soil microbial properties within each attribute was tentatively carried out taking into consideration the, from our own experience, proven capacity of the selected microbial parameters to provide useful information regarding the effects of soil disturbances [*e.g.*, agricultural practices (Mijangos et al., 2009; Muñoz-Leoz et al., 2011), sources of environmental stress (Epelde et al., 2012a), remediation treatments (Epelde et al., 2009c)] on soil functioning and, relevantly, on the specific attributes studied here according to

their abovementioned definitions. Nonetheless, depending on the specific interests, goals, expertise, resources, etc. of a given study, different soil microbial properties for the here presented attributes or other similar attributes of ecological relevance could certainly be used.

7.2.6 Statistical analysis

One- and two-way ANOVA and repeated measures ANOVA (using the Greenhouse-Geisser correction) (Greenhouse and Geisser, 1959) were conducted for data comparison on soil physicochemical and plant parameters obtained at different SL doses and sampling times using SPSS/PC Statistical Analysis Software (one-way ANOVA for comparing SL doses; repeated measures ANOVA for comparing sampling times; two-way ANOVA to check for the interaction between both factors). Fisher's PLSD-test was used to establish the significance ($P<0.05$) of the differences among means (results are labelled in Tables 7.2 and 7.3 only when, according to ANOVA, a significant interaction was found between SL dose and sampling time; otherwise, they are described in the text as percentages; see below). For microbial parameters, as the focus of the study was to assess the effect of SL dose, data were transformed as a percentage of the control value at each sampling time. Then, one-way ANOVAs were conducted for data comparison at different doses of SL. In addition, a variation partitioning analysis on microbial parameters and a principal component analysis (PCA) on absorbance values obtained with the Biolog Ecoplates™ after 50 h of incubation were performed using Canoco 5 (ter Braak and Šmilauer, 2012).

Regarding attributes of ecological relevance, soil vigour, stability, suppressiveness, organization and redundancy were calculated from data on soil microbial parameters, according to the following formula, based on the treated-soil quality index (T-SQI) proposed by Mijangos et al. (2010):

$$Attribute = 10^{\log m + \frac{\sum_{i=1}^{n} (\log n_i - \log m)}{n}}$$

where m is the control value (set to 100%) and n corresponds to the measured values for each parameter as a percentage of the control value. This index is appropriate for the assessment of soil quality in those cases where the soil has been intentionally treated to increase some parameters, as done in this study. When evaluating the effects of treatments, the T-SQI takes into account not only the magnitude of the change but also the direction (increase/decrease) of such change, unlike the index described by Bloem et al. (2006) which takes into consideration

only the magnitude of deviation of each parameter (using absolute values) from the reference value. The T-SQI also considers the maintenance of evenness among the studied parameters, including a new term in the numerator (see Mijangos et al., 2010). Since, in the present study, we were not interested in maintaining the evenness shown by the contaminated non-treated soil, such term was not included in the formula above.

The following parameters were selected for the studied attributes: (1) *vigour:* dehydrogenase activity, basal and substrate-induced respiration, microbial biomass C, ATP content and AWCD from Biolog EcoPlates™; (2) *stability:* values of the resistance (RS) and resilience (RL) indices from data on dehydrogenase activity and nitrification potential rate obtained during the soil stability test against heat stress; (3) *suppressiveness:* radial growth of *N. crassa*; (4) *organization:* Shannon´s diversity index from Biolog EcoPlates™ and real-time PCR and PCR-DGGE (number of bands) data for fungi, bacteria and archaea; and (5) *redundancy:* PCR-DGGE (number of bands) data for chitin-degrading and denitrifying bacteria. Then, *organization* has been estimated here from data on the function and structure of soil microbial communities (functional diversity of the heterotrophic cultivable portion of soil bacterial communities from Biolog EcoPlates™ data, and taxonomic structure of fungal, bacterial and archaeal communities from PCR data), while redundancy has been calculated from data on specific functions (chitin-degradation, denitrification) performed by soil microbial communities, as we were interested in redundancy within functional groups. In this regard, *a priori*, the loss of a functionally redundant species should have very little impact, or none at all, on the functioning of the ecosystem (Naeem et al., 1995; Hunt and Wall, 2002).

Finally, the arithmetic mean of the five attributes was calculated as index of soil quality (SQI):

SQI = [(vigour + stability + suppressiveness + organization + redundandy) / 5]

7.3 Results

7.3.1 Soil physicochemical parameters

As abovementioned, one- and two-way ANOVA and repeated measures ANOVA were conducted for data comparison on soil physicochemical parameters. Fisher's PLSD-test was used to establish the significance of the differences among means: results are labelled in Tables 7.2 and 7.3 only when, according to ANOVA, a significant interaction was found between SL dose and sampling time; otherwise, they are described in the text as percentages.

Regarding $CaCl_2$-extractable metal concentrations in soil (Table 7.2), one and six months after the application of 40 and 80 t SL ha^{-1}, a significant ($P<0.05$) reduction in bioavailable Cd

Table 7.2. $CaCl_2$-extractable metal concentrations (mg kg^{-1} DW) in soil before the application of sewage sludge-SL (initial), and one and six months after such application. Mean values (n = 3) ± SE. Sampling times labelled with different letters are significantly (P<0.05) different according to Fisher's PLSD-test (statistics are shown only when, according to ANOVA, a significant interaction was found between SL dose and sampling time).

	Cd			Pb			Zn		
	Initial	**1 month**	**6 months**	**Initial**	**1 month**	**6 months**	**Initial**	**1 month**	**6 months**
Control	0.28 ± 0.01^{a}	0.40 ± 0.06^{a}	0.46 ± 0.07^{a}	4.59 ± 0.60	3.08 ± 0.26	2.39 ± 0.00	29.05 ± 2.40	24.67 ± 4.08	32.11 ± 6.48
20 t SL ha^{-1}	0.46 ± 0.05^{a}	<0.25^{b}	0.37 ± 0.05^{ab}	3.16 ± 0.13	3.00 ± 0.07	3.81 ± 0.00	28.80 ± 4.91	15.80 ± 0.32	32.61 ± 4.36
40 t SL ha^{-1}	0.35 ± 0.02^{a}	<0.25^{b}	0.27 ± 0.01^{b}	3.00 ± 0.20	3.23 ± 0.36	<2	25.73 ± 2.99	17.95 ± 0.42	17.33 ± 4.78
80 t SL ha^{-1}	0.44 ± 0.04^{a}	<0.25^{b}	<0.25^{b}	3.68 ± 0.25	3.88 ± 0.43	2.44 ± 0.16	26.45 ± 2.05	14.81 ± 1.95	14.49 ± 1.60

F probabilities from two-way ANOVA and repeated measures ANOVA (Greenhouse-Geisser correction) (ns: non significant; *, ** and *** represent significancies of P<0.05, 0.01 and 0.001, respectively) for metals from left to right: Dose, ns, ns, ns; Time, **, **, **; Dose x Time, **, ns, ns

Table 7.3. Soil pH, water-soluble organic carbon (WSOC) and ammonium ($N\text{-}NH_4^+$) content before the application of sewage sludge-SL (initial), and one and six months after such application. Mean values (n = 3) ± SE. Sampling times labelled with different letters are significantly (P<0.05) different according to Fisher's PLSD-test (statistics are shown only when, according to ANOVA, a significant interaction was found between SL dose and sampling time).

	pH			WSOC (mg C kg^{-1})			$N\text{-}NH_4^+$ (mg kg^{-1})		
	Initial	**1 month**	**6 months**	**Initial**	**1 month**	**6 months**	**Initial**	**1 month**	**6 months**
Control	6.5 ± 0.1^{a}	6.8 ± 0.0^{b}	6.3 ± 0.1^{a}	99 ± 19	15 ± 5	156 ± 80	1.1 ± 0.4^{a}	1.2 ± 0.3^{a}	2.3 ± 0.1^{a}
20 t SL ha^{-1}	6.6 ± 0.1^{ab}	6.9 ± 0.2^{a}	6.2 ± 0.1^{b}	70 ± 3	67 ± 12	323 ± 21	2.5 ± 0.9^{a}	9.8 ± 1.8^{b}	2.1 ± 0.7^{a}
40 t SL ha^{-1}	6.5 ± 0.1^{a}	7.4 ± 0.3^{b}	6.2 ± 0.1^{a}	103 ± 19	198 ± 20	345 ± 47	1.6 ± 0.6^{a}	8.7 ± 1.2^{b}	2.9 ± 0.2^{a}
80 t SL ha^{-1}	6.6 ± 0.0^{a}	8.0 ± .00^{b}	5.9 ± 0.0^{c}	63 ± 12	312 ± 56	398 ± 107	2.7 ± 0.9^{a}	5.8 ± 0.1^{b}	7.0 ± 0.4^{b}

F probabilities from two-way ANOVA and repeated measures ANOVA (Greenhouse-Geisser correction) (ns: non significant; *, ** and *** represent significancies of P<0.05, 0.01 and 0.001, respectively) for parameters from left to right: Dose, ns, ns, ns; Time, ***, **, ***; Dose x Time, *, ns, **.

was observed, compared to initial values. Similarly, one month after the application of 20 t SL ha^{-1}, a significant reduction in bioavailable Cd was observed. Irrespective of SL dose, at the last sampling time (month 6), an overall significant 23% reduction in bioavailable Pb was observed, compared to the mean of values found at time 0 and one month. In turn, an overall significant 33% reduction in bioavailable Zn was observed one month after SL application; however, this significant difference was not observed at the last sampling time.

Pertaining to other soil physicochemical parameters (Table 7.3), one month after SL application, values of pH were significantly higher in soils treated with 40 and 80 t SL ha^{-1}, compared to initial values; by contrast, at month 6, lower pH values were observed in soils treated with 80 t SL ha^{-1}, compared to initial values (at this time, lower values of pH were found at month 6 *versus* month 1). Irrespective of SL dose, at the last sampling time, an overall significant 164% increase in WSOC was observed, compared to the mean of values found at time 0 and one month. Finally, at month 1, a significant increase in extractable N-NH_4^+ content was observed in all SL-treated soils, compared to initial values; at month 6, higher values of this parameter were only observed in soils treated with 80 t SL ha^{-1}.

7.3.2 Plant parameters

Control plants showed significantly higher values of shoot Cd concentration than plants growing in soils treated with 40 and 80 t SL ha^{-1}. Besides, plants growing in SL-treated soils showed significantly lower values of shoot Pb and Zn concentration (on average, 71 and 55% lower, respectively) than control plants. Regarding plant biomass, significantly higher values of this parameter were found in soils treated with 40 t SL ha^{-1} *versus* control soil (Table 7.4).

Table 7.4. Effect of treatments on shoot metal concentration (mg kg^{-1}) and plant biomass (g m^{-2}) six months after the application of sewage sludge (SL). Mean values (n = 3) ± SE. Sewage sludge doses labelled with different letters are significantly (P<0.05) different according to Fisher's PLSD-test.

	Cd	**Pb**	**Zn**	**Biomass**
Control	4.8 ± 0.4^{a}	407.2 ± 30.6^{a}	15.4 ± 1.2^{a}	31.7 ± 3.0^{a}
20 t SL ha^{-1}	3.6 ± 0.1^{ab}	147.3 ± 13.5^{b}	7.7 ± 0.4^{b}	60.1 ± 0.3^{ab}
40 t SL ha^{-1}	2.2 ± 0.1^{b}	118.6 ± 5.0^{b}	6.7 ± 0.5^{b}	103.6 ± 23.1^{b}
80 t SL ha^{-1}	2.8 ± 0.1^{b}	84.5 ± 14.0^{b}	6.6 ± 1.0^{b}	67.4 ± 6.8^{ab}

7.3.3 Soil microbial parameters

Microbial parameters are known to be highly dependent on the specific environmental conditions present at sampling time. In fact, according to the variation partitioning analysis, sampling time accounted for 83% of the explained variation in the observed data, while SL dose accounted for only 17% of the explained variation. As the focus of the study was to assess the effect of SL dose, data were transformed as a percentage of the control value at each sampling time

Just before SL application (initial values), apart from dehydrogenase activity, all microbial parameters used here to estimate soil vigour did not show significant differences among the studied plots (Figure 7.1), indicating that the experimental area was fairly homogeneous. At month 1, a significant increase in dehydrogenase activity, basal respiration, substrate-induced respiration and AWCD was found in plots treated with 40 and 80 t SL ha^{-1}, Just before SL application (initial values), apart from dehydrogenase activity, all microbial parameters used here to estimate soil vigour did not show significant differences among the studied plots (Figure 7.1), indicating that the experimental area was fairly homogeneous. At month 1, a significant increase in dehydrogenase activity, basal respiration, substrate-induced respiration and AWCD was found in plots treated with 40 and 80 t SL ha^{-1}, compared to control soil. At month 6, a significant increase in dehydrogenase activity, substrate-induced respiration, AWCD and ATP content was found in plots treated with 20 t SL ha^{-1} (higher values of dehydrogenase activity and AWCD were also observed in soils treated with 80 t SL ha^{-1}), compared to controls. On the contrary, no significant differences among treatments at any of the sampling times were observed for microbial biomass C

Regarding soil organization parameters (Figure 7.2), just before SL application, some significant differences were observed among the studied plots in terms of fungal gene abundance, bacterial gene abundance and number of fungal DGGE bands. However, at month 1 and 6, no significant differences among treatments were found for these three parameters. At month 1, no significant differences among treatments were observed for number of bacterial and archaeal DGGE bands. On the contrary, at month 1, the application of 80 t SL ha^{-1} led to higher values of archaeal gene abundance, compared to controls. As far as functional diversity (from Biolog Ecoplates™ data) is concerned, higher values of the Shannon´s diversity index were, in general, observed in SL-treated versus untreated soils at month 1 and month 6 (Figure 7.3a). On the other hand, the PCA performed on Biolog Ecoplates™ data (Figure 7.3b) did not separate treatments into clear-cut groups.

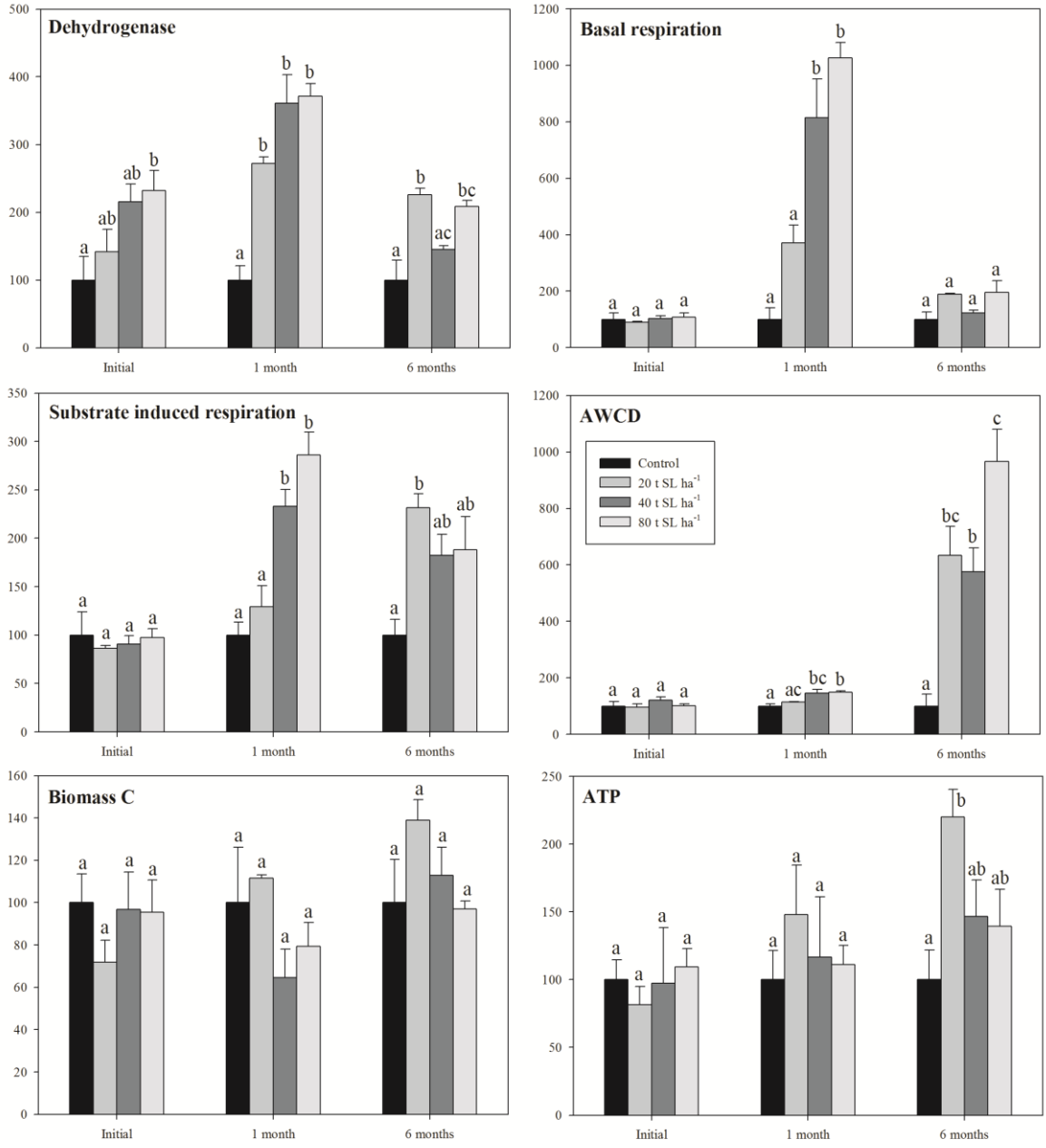


Figure 7.1. Effect of treatments on microbial parameters used to determine soil vigour (dehydrogenase activity, basal and substrate-induced respiration, average well colour development-AWCD, ATP content, microbial biomass C) before the application of sewage sludge-SL (initial), and one and six months after such application. Values are expressed as a percentage of the control at each sampling time. Mean values (n = 3) ± SE. Bars labelled with different letters at each sampling time are significantly ($P<0.05$) different according to Fisher's PLSD-test.

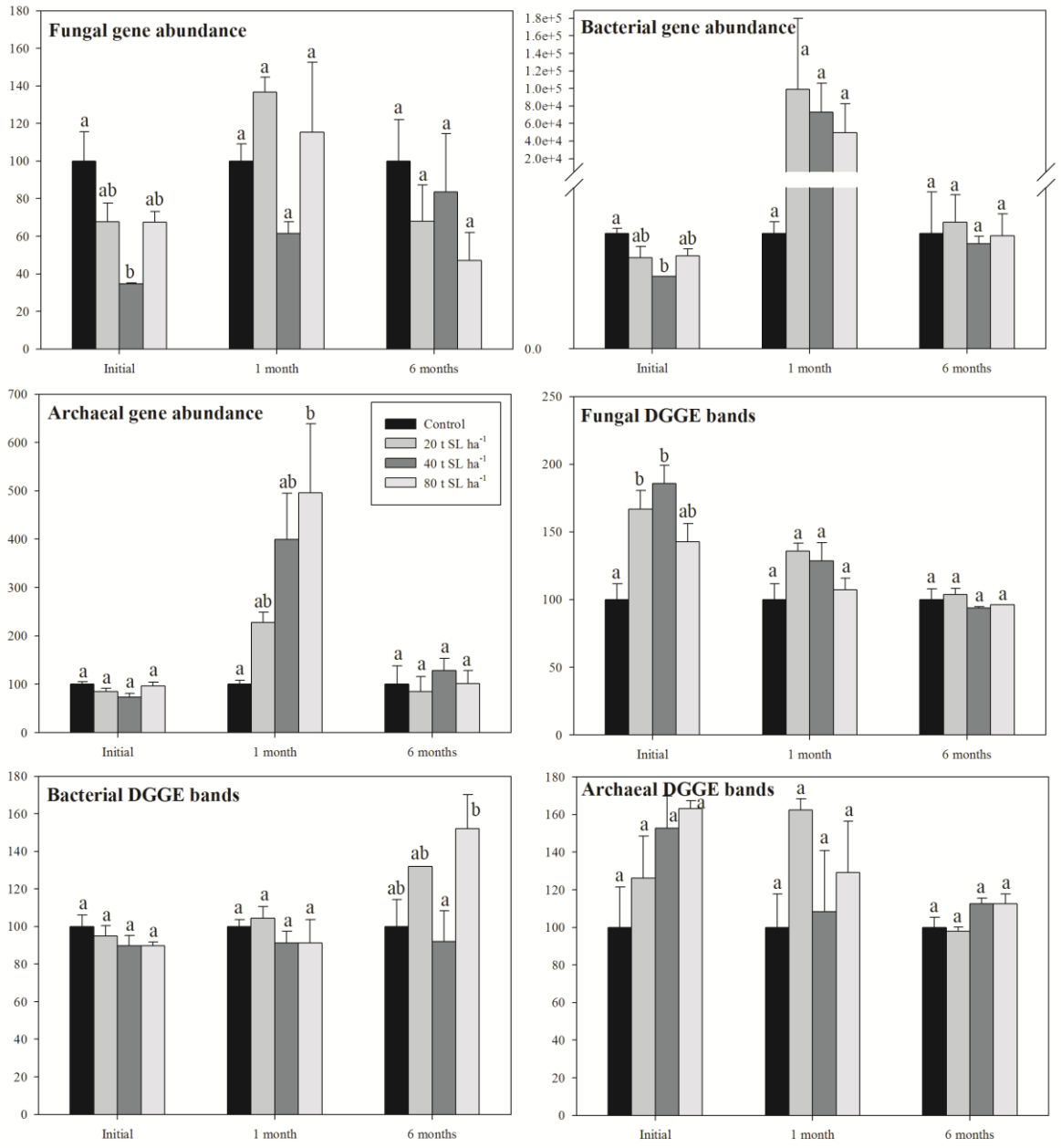
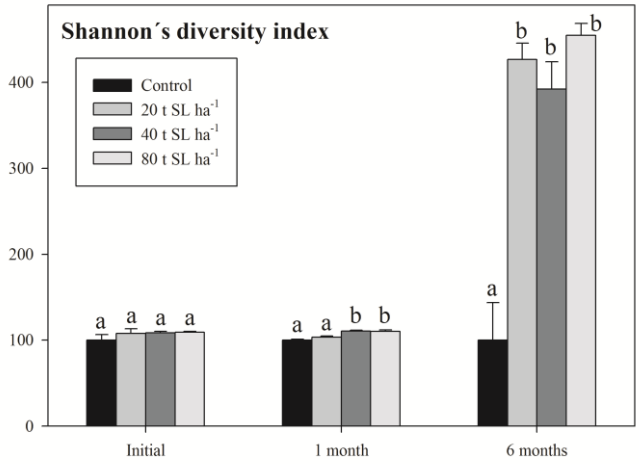


Figure 7.2. Effect of treatments on some microbial parameters used to determine soil organization (fungal, bacterial and archaeal gene abundance and number of DGGE bands) before the application of sewage sludge-SL (initial), and one and six months after such application. Values are expressed as a percentage of the control at each sampling time. Mean values (n = 3) ± SE. Bars labelled with different letters at each sampling time are significantly (P<0.05) different according to Fisher's PLSD-test.

(a)



(b)

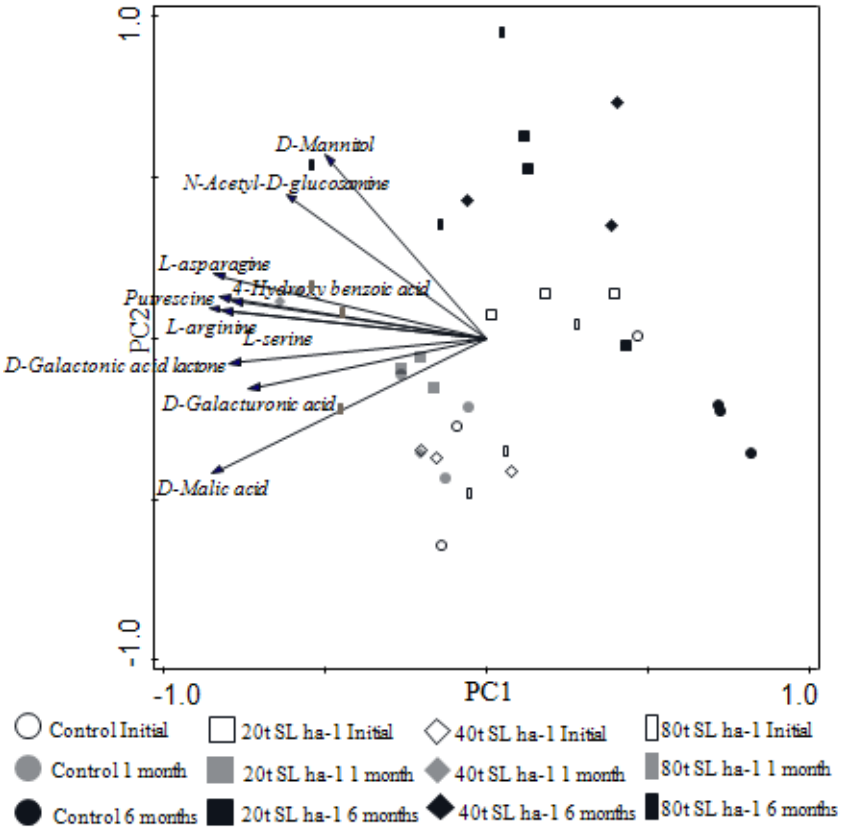


Figure 7.3. (a) Effect of treatments on Biolog EcoPlates™ data used to determine soil organization (*i.e.*, values of the Shannon´s diversity index) before the application of sewage sludge-SL (initial), and one and six months after such application. Values are expressed as a percentage of the control at each sampling time. Mean values (n = 3) ± SE. Bars labelled with different letters at eachsampling time are significantly (P<0.05) different according to Fisher's PLSD-test. (b) Principal component analysis from absorbance values obtained with the Biolog EcoPlates™; PC1 and PC2 account for 51 and 12% of the variance, respectively. The 10 carbon substrates showing the best fit are represented with arrows

As far as soil stability is concerned (Figure 7.4), at month 6, significantly higher values of the RS index calculated from data on dehydrogenase activity and the RL index calculated from data on nitrification potential rate were observed in soils treated with 40 and 80 t SL ha^{-1} (and lower values of the RS index calculated from nitrification potential rate in soils treated with 80 t SL ha^{-1}), compared to control soil.

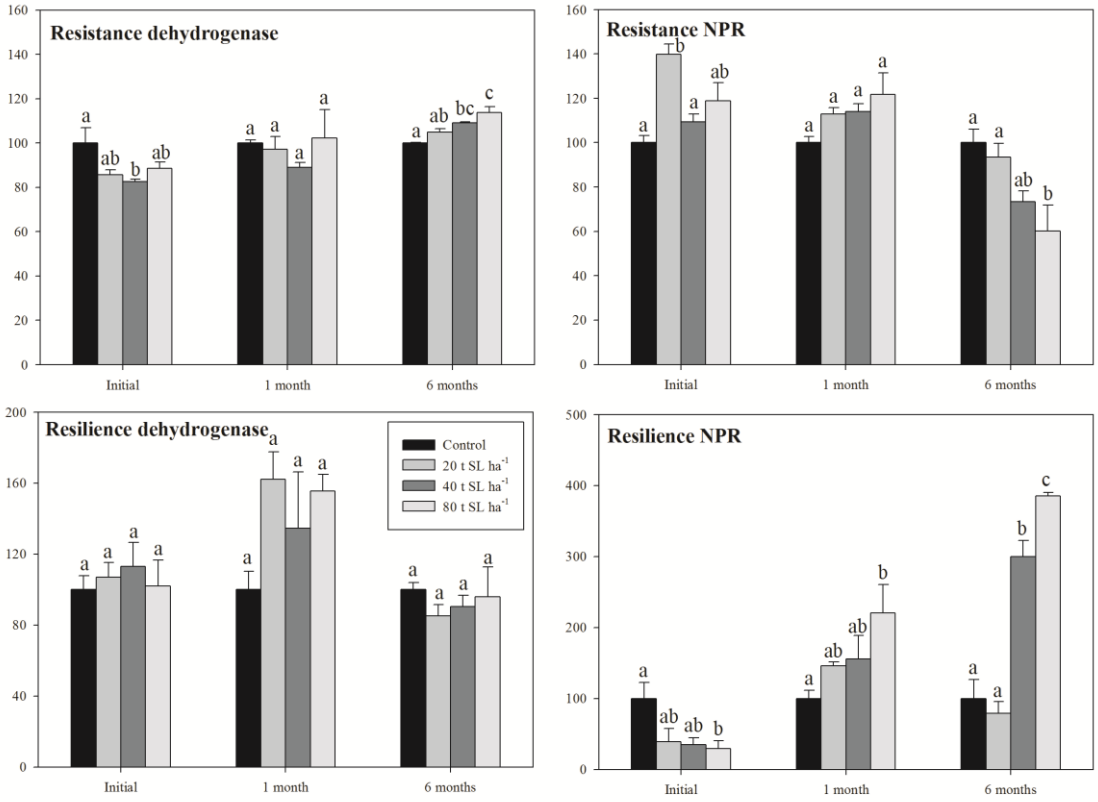


Figure 7.4. Effect of treatments on microbial parameters used to determine soil stability (resistance and resilience indices from data on dehydrogenase activity and nitrification potential rate-NPR) before the application of sewage sludge-SL (initial), and one and six months after such application. Values are expressed as a percentage of the control at each sampling time. Mean values (n = 3) ± SE. Bars labelled with different letters at each sampling time are significantly (P<0.05) different according to Fisher's PLSD-test.

Finally, no significant differences were found among treatments at any of the sampling times for those parameters used here to estimate soil redundancy (number of chitin-degrading and denitrifying DGGE bands) (Figure 7.5) and suppressiveness (Figure 7.6), compared to controls.

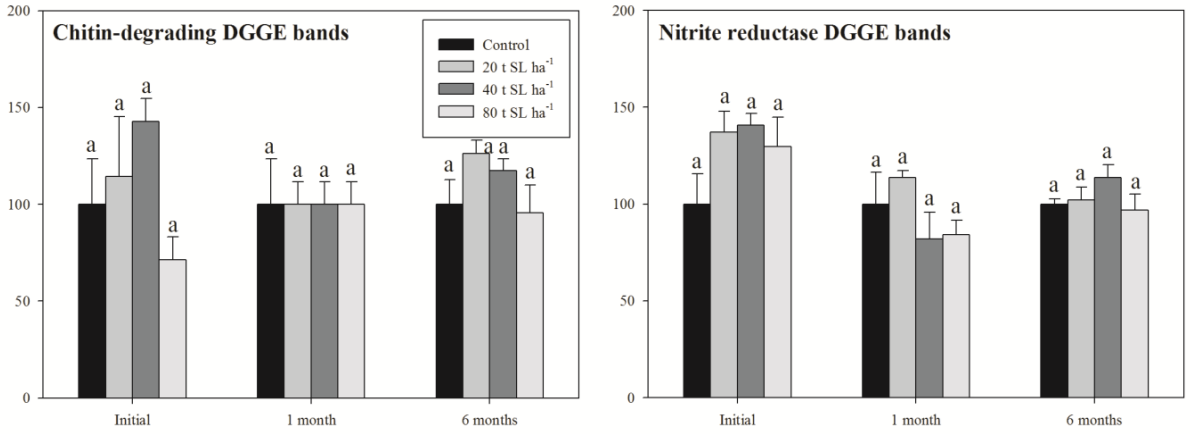


Figure 7.5-. Effect of treatments on microbial parameters used to determine soil redundancy (number of DGGE bands for chitin-degrading and denitrifying bacteria) before the application of sewage sludge-SL (initial), and one and six months after such application. Values are expressed as a percentage of the control at each sampling time. Mean values (n = 3) ± SE. Bars labelled with different letters at each sampling time are significantly (P<0.05) different according to Fisher's PLSD-test.

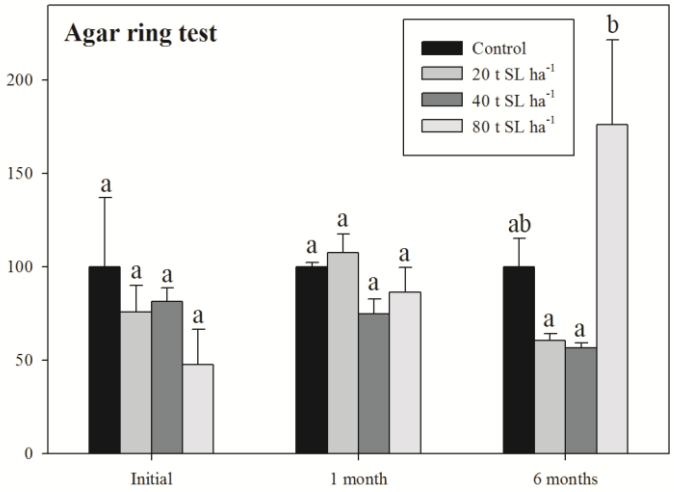


Figure 7.6-. Effect of treatments on microbial parameters used to determine soil suppressiveness (agar ring test with *Neurospora crassa*) before the application of sewage sludge-SL (initial), and one and six months after such application. Values are expressed as a percentage of the control at each sampling time. Mean values (n = 3) ± SE. Bars labelled with different letters at each sampling time are significantly (P<0.05) different according to Fisher's PLSD-test.

7.3.4 Attributes of ecological relevance

Regarding the values of soil attributes calculated from data on microbial parameters (Table 7.5), at month 1, values of vigour were significantly higher in soils treated with 40 and 80 t SL ha^{-1} compared to the control. Similarly, at month 6, values of vigour were significantly higher in soils treated with 20 and 80 t SL ha^{-1}. After 1 month of treatment, higher values of

Table 7.5-. Effect of treatments on attributes of ecological relevance and soil quality before the application of sewage sludge-SL (initial), and one and six months after such application. Values are expressed as a percentage of the control at each sampling time. Mean values (n = 3) ± SE. Values labelled with different letters at each sampling time are significantly (P<0.05) different according to Fisher's PLSD-test.

		Vigour	Organization	Stability	Redundancy	Suppressivenss	Quality
Initial	**Control**	100 ± 21^{a}	100 ± 2^{ab}	100 ± 6^{a}	100 ± 3^{a}	100 ± 6^{a}	100 ± 3^{a}
	20 t SL ha^{-1}	92 ± 9^{a}	100 ± 3^{ab}	85 ± 7^{a}	108 ± 5^{ab}	97 ± 4^{a}	97 ± 1^{a}
	40 t SL ha^{-1}	112 ± 20^{a}	91 ± 3^{a}	83 ± 4^{a}	115 ± 1^{b}	99 ± 2^{a}	100 ± 4^{a}
	80 t SL ha^{-1}	119 ± 14^{a}	104 ± 1^{b}	80 ± 5^{a}	100 ± 1^{a}	88 ± 6^{a}	98 ± 2^{a}
1 month	**Control**	100 ± 21^{a}	100 ± 4^{a}	100 ± 1^{a}	100 ± 8^{a}	100 ± 0^{a}	100 ± 2^{a}
	20 t SL ha^{-1}	173 ± 12^{ab}	244 ± 72^{a}	118 ± 2^{b}	104 ± 2^{a}	101 ± 2^{a}	148 ± 17^{b}
	40 t SL ha^{-1}	204 ± 31^{b}	259 ± 42^{a}	111 ± 8^{ab}	98 ± 3^{a}	95 ± 2^{a}	153 ± 13^{b}
	80 t SL ha^{-1}	234 ± 6^{b}	268 ± 23^{a}	126 ± 1^{b}	98 ± 2^{a}	97 ± 3^{a}	165 ± 6^{b}
6 months	**Control**	100 ± 30^{a}	100 ± 31^{a}	100 ± 5^{ab}	100 ± 2^{ab}	100 ± 3^{ab}	100 ± 13^{a}
	20 t SL ha^{-1}	251 ± 13^{b}	138 ± 18^{a}	94 ± 4^{a}	104 ± 0^{a}	92 ± 1^{a}	136 ± 3^{b}
	40 t SL ha^{-1}	185 ± 14^{ab}	140 ± 11^{a}	116 ± 3^{b}	105 ± 1^{a}	91 ± 1^{a}	127 ± 4^{ab}
	80 t SL ha^{-1}	225 ± 28^{b}	139 ± 15^{a}	118 ± 7^{b}	98 ± 1^{b}	108 ± 6^{b}	138 ± 7^{b}

stability were found in soils treated with 20 and 80 t SL ha^{-1}. In contrast, at month 1 and 6, no significant differences in organization, redundancy and suppressiveness were detected for any of the treatments, compared to controls. Finally, at month 1, a significant improvement in soil quality was observed in all SL-treated soils; likewise, at month 6, a significant improvement in soil quality was detected in soils treated with 20 and 80 t SL ha^{-1}

7.4 Discussion

7.4.1 Soil physicochemical and plant parameters as indicators of treatment effectiveness

In this study, the effect of treatments on soil metal bioavailability has been estimated through the determination of $CaCl_2$-extractable metal fractions in soil as well as shoot metal concentrations. Conflicting reports can be found in the scientific literature concerning the effectiveness of OM amendments for soil metal binding and, most importantly, whether such amendments serve as a source or sink for metals. In this respect, a critical factor is the source of the organic amendment (O'Dell et al., 2007). Metal concentrations in our experimental mine soil are extremely high; therefore, the incorporation of a small amount of metal to the soil through the application of SL caused, most likely, no additional adverse impact on the soil ecosystem.

A very low fraction of total metal concentrations in soil was extractable with $CaCl_2$. A reduction in $CaCl_2$-extractable metal concentration in soil due to SL addition was clearly observed only for Cd, which is one of the most deleterious trace metals to living organisms (Dong et al., 2007). On the contrary, SL addition led to a significant reduction in shoot metal concentration for the three metals under study, which could be due to reduction in metal availability and/or metal dilution in plant tissues due to plant growth. Although $CaCl_2$-extractable metal fractions are thought to reflect metal bioavailability in soil (Houba et al., 2000), Smith et al. (2010) found $CaCl_2$-extractable metal fractions not to be correlated with biological responses of *Eisenia andrei* (earthworm), *Folsomia candida* (collembola), *Elymus lanceolatus* (northern wheatgrass) and *Medicago sativa* L. (alfalfa). The vegetation naturally growing in our experimental area was dominated by *Festuca rubra* L., previously identified as a metal excluder in our mining site (Barrutia et al., 2011). Regrettably, there is still no standardized chemical method for the measurement of metal bioavailability in soil remediation monitoring programs.

In another regard, an increase in soil pH was indeed observed at month 1 due to the addition of 40 and 80 t SL ha^{-1}, but this beneficial effect (in terms of the expected concomitant reduction in metal bioavailability and mobility) was transient; actually, in soil treated with 80 t SL ha^{-1}, at month 6, values of pH were even lower than initial values measured before SL addition. Similarly, the higher values of $N\text{-}NH_4^+$ content found at month 1 in soils treated with 20 and 40 t SL ha^{-1} were also transient. As described above, the incorporation of organic amendments to metal contaminated soil during chemical stabilization is aimed at reducing metal availability and improving soil quality through an increase in soil pH, OM content, nutrients, etc. Nonetheless, as stated by Tordoff et al. (2000), the use of chemical methods, such as chemical stabilization, for soil remediation appears restricted by their lack of permanency and the need for regular inspections.

7.4.2 Soil microbial parameters as indicators of treatment effectiveness

Although not the focus of the present study, it is important to always remember that sampling time has a strong influence on soil microbial parameters (Nielsen et al., 2009; Sugihara et al., 2010). Then, here it must be taken into account that soil samplings were performed in different seasons (the first two in winter and the third one in summer).

Pertaining to the effect of treatments on soil microbial properties, SL addition had a more pronounced effect on parameters used here to estimate soil vigour. Several studies have reported an increase in microbial activity in metal-contaminated soils as a result of the addition of SL and other organic amendments (Sastre et al., 1996; Moreno et al., 2002; Tejada et al., 2008). This increase in microbial activity was probably due to the reduction in metal (mainly, Cd) bioavailability and also, at least partly, to the input of easily biodegradable OM resulting from the application of SL. Out of the six parameters analyzed here to determine soil vigour (Figure 7.1), those that depend on the activity of living cells (dehydrogenase activity, basal respiration, substrate-induced respiration, AWCD, ATP) showed some significant increase as a result of SL addition. On the other hand, the pool of non-active cells present in the soil could be contributing to the lack of significant differences in microbial biomass C values observed during the 6-month monitoring period. A longer monitoring period might be required to detect significant changes in microbial biomass C, compared to, for instance, values of substrate-induced respiration (an indicator of active microbial biomass) (Hassink, 1993).

Some significant differences between SL-treated and control soils were also observed for values of RS and RL indices. In any case, different results were obtained depending on the specific parameter used to calculate the effect of treatments on RS and RL indices: dehydrogenase activity *versus* nitrification potential rate. This is not surprising as dehydrogenase activity is an indicator of overall microbial activity in soil (Nannipieri et al., 2002), while nitrification potential rate targets a more specialized biological process. Interestingly, SL-induced changes in RS and RL indices were mainly observed at the end of the experiment, indicating a slower response to the introduced disturbance (SL addition), as compared to vigour parameters.

Likewise, in general, no clear significant differences were observed among SL-treated and control soils regarding those parameters used here to determine soil redundancy and suppressiveness. Ghini et al. (2007) reported that the effect of SL on soil suppressiveness depends on the specific pathogen, methodology applied, and time interval between SL addition and soil sampling. Although SL had a clear effect on microbial activity (vigour), it did not significantly alter the composition of the soil microbial communities as shown by PCR-DGGE data. As stated above for microbial biomass C values, the pool of non-active cells present in the soil could be contributing to the lack of significant differences in PCR-DGGE data observed during the 6-month monitoring period. At month 1 and month 6, the functional diversity of soil microbial communities (as reflected by the values of H' from Biolog Ecoplates™ data) was significantly affected by treatments; by contrast, the addition of SL did not result in any significant changes in the structural diversity of soil microbial communities (from PCR-DGGE data). This is not surprising as Biolog™ plates reflect the potential of the heterotrophic cultivable portion of the soil bacterial community to utilize C sources (Hitzl et al., 1997), while PCR-DGGE bands provide a genetic profile of the dominant microbial species (Marzorati et al., 2008). Although these techniques are certainly biased towards certain fractions of the soil microbial community, this should, however, not question their applicability, since macroecologists also successfully work on diversity aspects of subfractions of the total community (Sharma et al., 1998). Functional Biolog™ diversity of soil bacterial communities has also been reported to increase as a result of other organic (maize litter) amendments (Sharma et al., 1998).

7.4.3 Soil attributes of ecological relevance as indicators of treatment effectiveness

Following Garbisu et al. (2011), we have linked the concept of soil quality to that of ecosystem health (*sensu* Rapport, 1998) through the grouping of soil microbial properties within a set of ecosystem attributes of ecological relevance: (i) *vigour*, operationally evaluated in terms of productivity, or throughput of material or energy in the system; (ii) *organization*, quantified in terms of diversity of components and their degree of mutual dependence; (iii) *stability*, assessed in terms of the system's ability to maintain its structure and pattern of behaviour in the presence of a disturbance; (iv) *suppressiveness* (suppressive soils are soils in which disease incidence remains low, despite the presence of a pathogen, a susceptible host plant, and climatic conditions favourable for disease development) (Janvier et al., 2007), as an important characteristic of a good quality soil (van Bruggen and Semenov, 2000); and (v) *redundancy*, a most relevant attribute as ecosystem functions may not be affected by the loss of a species from an ecosystem if other species are able to perform the same function.

Interestingly, when soil microbial properties are grouped within attributes of ecological relevance, a complementary interpretation of the effects of treatments on soil quality can be performed. In this regard, at the end of the experiment, the effect of SL addition on ecosystem attributes of ecological relevance was only observed for soil vigour, which is related with the activity, metabolism, or primary productivity of an ecosystem. As stated above, the SL-induced increase in soil vigour is probably due to the reduction in metal (mainly, Cd) bioavailability and also, at least partly, to the input of easily biodegradable OM resulting from the application of SL (Alvarenga et al., 2009a; 2009b). In the short term (month 1), an improvement in soil stability was also detected in soils treated with 20 and 80 t SL ha^{-1}; unfortunately, this improvement was transient as no significant differences in stability were observed at month 6 between SL-treated and control soils. In this respect, no differences in biodiversity, as reflected by the number of fungal, bacterial and archaeal bands in the DGGE analysis, was observed between SL-treated and control soils at month 6. The connection between biodiversity and ecosystem stability has been the subject of a long-standing debate in ecology. Nowadays, there is mounting evidence that biodiversity increases the stability of ecosystem processes through time (Tilman et al., 2006; Griffin et al., 2009; Jiang and Pu, 2009; Campbell et al., 2011; Loreau and de Mazancourt, 2013); however, the underlying mechanisms (*e.g.*, asynchrony of species´ intrinsic responses to environmental fluctuations, differences in the speed at which species respond to perturbations, reduction in the strength of competition) are still poorly understood

(Loreau and de Mazancourt, 2013). In any case, stability is an emergent property of an ecosystem, coming from the interactions between the constituent species, instead of a property of the species themselves.

Most importantly, when calculating soil quality from the five attributes determined here, a SL-induced improvement in soil quality was observed at both month 1 and month 6. Then, in our metal contaminated mine soil, chemical stabilization with lime-treated SL was only partly successful: it did improve soil quality but the expected reduction in soil metal bioavailability (as reflected by the values of $CaCl_2$-extractable metal concentration) was clearly observed only for Cd (not for Pb or Zn).

Finally, the interpretation of data on soil microbial parameters within a set of ecosystem attributes of ecological relevance should facilitate the communication between scientists and non-scientist stakeholders. Hopefully, when dealing with data on soil quality, soil managers, decision makers and alike will possibly feel more comfortable with terms such as vigour, organization, stability, redundancy, etc., than with terms such as dehydrogenase activity, substrate-induced respiration, nitrification potential rate, AWCD, etc. (Garbisu et al., 2011). Furthermore, the utilization of attributes of ecological relevance could provide long-term soil quality monitoring programs with the required stability over time, so that they will not be affected by the changes in methods, equipments, and interests which may occur during the monitoring program. In any event, as occurs with the use of indices, it is important to take into account that this approach inevitably results in information compression and can lead to an oversimplification of available information; in consequence, when possible, it is desirable to assess soil quality at both levels: the indicator (microbial parameter) level and the attribute level, since they provide complementary information.

08 | SÍNTESIS

8. SÍNTESIS

8.1 Impacto y remediación de los contaminantes

El suelo es un sistema vivo, dinámico y complejo, cuyas funciones son de gran importancia para el mantenimiento de los ecosistemas terrestres y nuestra propia supervivencia; sin embargo, en la sociedad moderna, la tendencia mayoritaria ha sido a considerarlo como un componente inanimado de la Tierra. Esta situación ha propiciado la liberación en el suelo de una gran cantidad de sustancias químicas contaminantes, las cuales, y en particular los metales pesados, han generado un problema medioambiental de gran magnitud, con efectos devastadores a escala global sobre la funcionalidad y sostenibilidad del ecosistema edáfico. La contaminación de suelos por metales pesados debido a actividades antropogénicas (*e.g.*, industria, minería y agricultura) suele generarse como resultado de diversos episodios contaminantes de uno o, más habitualmente, varios metales simultáneamente (Mertens y cols., 2006). No obstante, la mayoría de estudios sobre el impacto de la contaminación de suelos por metales pesados, los cuales se basan en contaminar artificialmente un suelo y posteriormente evaluar el impacto, se efectúan mediante la aplicación de un único episodio contaminante y, generalmente, con un único metal pesado. Asimismo, existen muchos estudios sobre el impacto de los metales pesados en suelos ya contaminados, pero, en estos casos, un problema importante radica en la ausencia de datos sobre la calidad del suelo relativa al estado previo a la contaminación, o a la ausencia de un suelo similar no-contaminado que sirva de referencia.

En este contexto, decidimos investigar el impacto que tiene la contaminación reiterada por metales pesados sobre el suelo, en términos de efectos tóxicos, con el objetivo de simular, en la medida de lo posible, las condiciones que más frecuentemente se dan en los casos de contaminación de suelos por metales pesados y evaluar así dicho impacto a lo largo del tiempo. Respecto a la evaluación de la toxicidad de los metales pesados presentes en el suelo, teniendo en cuenta que el mayor riesgo para los seres vivos y para el funcionamiento del ecosistema edáfico es producido por la fracción biodisponible (Kumpiene y cols., 2009), la concentración de metales pesados biodisponibles se presenta como un buen indicador del impacto de contaminación metálica. Si bien no hay un método estandarizado para la medición de metales pesados biodisponibles en los programas de monitorización de suelos contaminados, diversos autores coinciden en que los extractantes salinos, y en particular el $CaCl_2$, son los métodos más idóneos para reflejar y predecir la biodisponibilidad de los metales

pesados en el suelo y su potencial efecto toxico (Houba y cols., 2000; Madejón y cols., 2006; Menzies y cols., 2007).

Así pues, en el Capítulo 4, se contaminó de forma artificial un suelo mediante la aplicación de metales pesados (Cd, Pb, Cu y Zn) en tres episodios sucesivos, tanto de forma individual como conjunta, y se estimó la concentración de metales pesados biodisponibles en el suelo tras cada episodio contaminante. Los valores de metales pesados extraíbles en $CaCl_2$ confirmaron un hecho en absoluto sorprendente: no existe una correlación entre las concentraciones totales y las concentraciones biodisponibles. La biodisponibilidad de los metales pesados en el suelo suele estar fuertemente influenciada por una serie de factores, como pH y contenido de materia orgánica y arcillas (Lamb y cols., 2009). Además, distintos metales pesados tienen distinta afinidad por la materia orgánica, arcillas, etc., por lo que cada metal pesado presenta distintos mecanismos de sorción/desorción y, por lo tanto, distinta biodisponibilidad en el suelo. En nuestro estudio, los cuatro metales pesados empleados pueden ser clasificados de acuerdo a su biodisponibilidad de la siguiente manera (de menor a mayor): Cu ~ Pb < Zn < Cd.

Además, en nuestro ensayo se observó que el comportamiento de cada metal pesado cambiaba a lo largo del tiempo, ya que la biodisponibilidad relativa (fracción biodisponible respecto a la concentración total) aumentaba con los sucesivos episodios contaminantes, sobre todo cuando los metales pesados eran aplicados conjuntamente. Esto se puede deber, en parte a una descarga de carbono orgánico disuelto (COD), producida por un aumento de la mortalidad de los (micro)organismos del suelo, que posteriormente formaría organo-complejos solubles con los metales pesados (Giusquiani y cols., 1998); y, por otro lado, al aumento del "pool" de metales pesados solubles con cada episodio contaminante que saturarían superficies y ácidos húmicos disponibles en el suelo a los que poder adherirse, por lo que una mayor proporción de metales pesados permanecería en forma disponible.

Los metales en su forma biodisponible suponen un riesgo para los ecosistemas ya que presentan una gran probabilidad de ser lixiviados y/o entrar en la cadena trófica al ser incorporados por la biota del suelo (Madejón y cols., 2006). Así pues, en casos de contaminación de suelos por metales pesados, es fundamental llevar a cabo un proceso remediador para controlar y reducir su efecto tóxico. En este sentido, la utilización de soluciones basadas en la naturaleza, como la *fitorremediación* (el uso de plantas y sus microorganismos asociados), se presenta como una tecnología respetuosa con el medio

ambiente y con gran potencial de remediar suelos contaminados. Más concretamente, la *fitoextracción* (la utilización de especies vegetales capaces de extraer metales del suelo y acumularlos en sus tejidos, especialmente en las partes aéreas) presenta la ventaja de sustraer los metales pesados del suelo reduciendo considerablemente el riesgo de toxicidad.

Empero, la utilización de esta tecnología se encuentra con varias limitaciones debido a que las especies acumuladoras, aunque tolerantes a los metales pesados, suelen presentar en estos suelos contaminados una biomasa reducida y un crecimiento lento. Además, la biodisponibilidad de metales pesados en el suelo, especialmente en casuísticas de contaminación crónica, puede ser baja, lo que dificulta su incorporación por parte de las plantas. Esta situación obliga a explorar alternativas, que eliminen o, en su defecto, minimicen estas limitaciones, enfocadas principalmente a estimular la producción de biomasa vegetal y la toma de metales pesados durante la fitoextracción.

En este sentido, los entornos mineros albergan una biodiversidad única y característica, adaptada a las duras condiciones comúnmente presentes en este tipo de áreas, con un gran potencial para la fitorremediación y fitogestión de suelos contaminados por metales pesados (Barrutia y cols., 2011). Estas especies o variedades nativas de suelos metalícolas poseen ciertas características inherentes (*e.*g., tolerancia a los metales pesados, adaptación a la climatología y a las condiciones físico-químicas del suelo, etc.) que les confieren cierta ventaja sobre especies no-nativas en términos de colonización, crecimiento y acumulación de metales pesados en este tipo de ambientes. En el presente trabajo, se planteó explorar distintas opciones fitorremediadoras mediante la ejecución de una serie de estudios centrados en la mina de Lanestosa, un entorno minero que presenta una comunidad vegetal singular cuyas características ya han sido descritas en el Capítulo 2 (Procedimientos generales).

Así pues, en el Capítulo 5, se estudió a escala microcosmos la utilización de los ecotipos nativos de la mina de Lanestosa de dos especies metalófitas acumuladoras de metales, *Noccaea caerulescens* y *Rumex acetosa*, para la fitoextracción de un suelo contaminado. Así mismo, se hizo hincapié en valorar la utilización de ambas especies de forma combinada, comparándola con su uso de forma individual. La eficiencia de un proceso de fitoextracción depende de (i) la concentración de metales pesados en la parte aérea de la planta y (ii) la biomasa de la planta (Barrutia y cols., 2009). Al comparar los resultados obtenidos de estos parámetros, se corroboró lo observado en otros estudios sobre la coexistencia de varias especies metalófitas (Liu y cols., 2011; Yang y cols.,

2012; Lucisine y cols., 2014): la combinación de las dos especies acumuladoras tuvo un notable efecto positivo en la fitoextracción de metales pesados, resultando en un incremento tanto de la biomasa de las plantas (41 y 17% en *N. caerulescens* y *R. acetosa*, respectivamente, en comparación con la biomasa de estas especies obtenida de forma individual), como de la acumulación de metales pesados en sus tejidos, especialmente Zn. De hecho, la combinación de *N. caerulescens* y *R. acetosa* ya había sido sugerida por Epelde y cols. (2012b) como una opción óptima para la fitoextracción de Zn. Por otro lado, los tiestos con *N. caerulescens* mostraron valores más bajos de metales pesados biodisponibles, especialmente cuando esta especie se encontraba de manera individual. Es probable que las cinéticas de reposición de las fracciones metálicas biodisponibles no hayan podido compensar la elevada tasa de incorporación de metales pesados por parte de *N. caerulescens* (Whiting y cols., 2001).

Sin embargo, a pesar del evidente potencial fitoextractor de estas especies, y en especial de *N. caerulescens* (con valores de Zn por encima de 10.000 mg kg^{-1} en su parte aérea), esto no necesariamente repercutió en una reducción considerable de la concentración total de metales pesados en el suelo. Estimaciones de algunos autores (Zhao y cols., 2003; Ernst, 2005) del tiempo requerido para alcanzar una reducción de la concentración de metales pesados en el suelo que cumplan con los valores máximos establecidos por la legislación, o que al menos no conlleven un riesgo para los ecosistemas y la salud humana, sugieren que la fitoextracción no es una tecnología viable para suelos contaminados con elevadas concentraciones de metales pesados. En estos casos, la fitoestabilización mediante la revegetación con especies tolerantes se presenta como una opción idónea para este tipo de emplazamientos, ya que consigue reducir la biodisponibilidad de los metales en el suelo y, por tanto, controlar el riesgo de dispersión y ecotoxicidad.

En el Capítulo 6, se realizó un experimento a escala microcosmos de fitoestabilización con *Festuca rubra*, en el que se comparó el potencial fitoestabilizador de dos variedades distintas de esta especie metalófita exclusora: una variedad nativa de la mina de Lanestosa y una variedad comercial. Al final del experimento, la variedad comercial resultó severamente afectada por la toxicidad de los metales pesados, mostrando evidentes síntomas visuales de fitotoxicidad (*i.e.*, hojas cloróticas). Asimismo, la biomasa de la parte aérea en la variedad comercial fue un 48% menor que la obtenida por la variedad Lanestosa. Esto también se vio reflejado en el estado fisiológico de las plantas, puesto que la variedad comercial presentaba niveles menores

de carotenoides, clorofilas y eficiencia fotosintética (Fv/Fm), así como niveles mayores de tocoferol, lo que indica un estrés fisiológico. Además, los valores de metales pesados en los tejidos de las plantas de la variedad comercial (mucho mayores que los encontrados en la variedad Lanestosa) evidenciaron su incapacidad para controlar la toma y translocación de metales pesados. Todo esto demostró la superioridad de la variedad Lanestosa de *F. rubra*, respecto a la variedad comercial, debido a su capacidad de vencer los problemas asociados con el crecimiento en el suelo minero de Lanestosa.

La revegetación de suelos contaminados con especies metalófitas, además de mejorar las propiedades físico-químicas y biológicas del suelo, incrementando el contenido de materia orgánica, los niveles de nutrientes, la capacidad de intercambio catiónico y la actividad biológica (Arienzo y cols., 2004), estabiliza los metales pesados del suelo, evitando así su dispersión y movilidad. En este sentido, la variedad nativa de la mina de Lanestosa, con un sistema radicular mayor y más desarrollado, redujo la biodisponibilidad de metales pesados en el suelo, lo que demuestra que la utilización de especies metalófitas nativas de emplazamientos mineros, tolerantes a los metales pesados así como a otros factores ambientales adversos característicos de este tipo de emplazamientos, son una opción válida para la fitoestabilización.

Sin embargo, en muchas ocasiones, particularmente en entornos mineros, incluso la utilización de plantas tolerantes para revegetar suelos contaminados por metales pesados presenta una serie de limitaciones. Generalmente, los suelos mineros son ambientes poco favorables para la vida ya que, además de elevadas concentraciones de metales pesados, presentan bajos contenidos de materia orgánica y nutrientes, una elevada acidez y una estructura muy pobre (Wong, 2003), lo que dificulta ciertamente la colonización y el crecimiento de las plantas. La incorporación de enmiendas, orgánicas o inorgánicas, al suelo durante procesos fitoestabilizadores (*fitoestabilización inducida* o *quimiofitoestabilización*) ha demostrado ser una buena herramienta para facilitar la revegetación en entornos mineros ya que mejoran la estructura del suelo, aportan nutrientes y materia orgánica, y reducen la biodisponibilidad de los metales pesados (Alvarenga y cols., 2009a; 2009b)).

Así pues, en el ensayo de fitoestabilización con *F. rubra* del Capítulo 6, se decidió incluir la aplicación de purín de vaca con el objetivo de explorar el potencial de las enmiendas orgánicas y evaluar su eficiencia en procesos fitoestabilizadores. La aplicación de la enmienda resultó en una reducción de la biodisponibilidad de metales pesados en el suelo. Esta inmovilización de metales pesados, junto con la incorporación

de nutrientes como consecuencia de la adición de enmiendas orgánicas (Galende y cols., 2014b), contribuyó a aumentar la biomasa de las plantas y a reducir la translocación de metales pesados hacia las partes aéreas, resultando en una reducción de la concentración de metales pesados en estos tejidos de la planta. Así mismo, estas mejoras se reflejaron en los parámetros fisiológicos de las plantas: mayores niveles de carotenoides, clorofilas y eficiencia fotosintética (Fv/Fm) y menores niveles de tocoferol y ratio A+Z/VAZ.

Aunque el efecto positivo de la enmienda se observó en ambas variedades, la mejora fue mucho más evidente en la variedad comercial, lo cual es entendible teniendo en cuenta que esta variedad padecía más intensamente la fitotoxicidad por metales pesados. Además, es interesante señalar que el ratio "biomasa raíz: biomasa parte aérea" de la variedad Lanestosa era mayor en ausencia de enmienda que con enmienda. Este hecho indica que, incluso sin las ventajas aportadas por la enmienda orgánica, la variedad Lanestosa es capaz de presentar un sistema radicular más desarrollado lo que permite maximizar el potencial fitoestabilizador de esta especie, demostrando que la viabilidad de la variedad nativa Lanestosa de *F. rubra* es mucho mayor que la viabilidad de la variedad comercial.

A pesar de los beneficios aportados por las enmiendas orgánicas, su aplicación debe ser llevada a cabo con cuidado ya que conlleva una serie de riesgos ambientales, como la reducción de la diversidad microbiana, la contaminación de aguas subterráneas por nitratos, la proliferación de bacterias resistentes a antibióticos, o una inversión de la inmovilización de los metales pesados debido a la descomposición de la materia orgánica a lo largo del tiempo (Martínez y cols., 2003; Jorge-Mardomingo y cols., 2015). En la búsqueda de alternativas ambientalmente más respetuosas para optimizar procesos de fitorremediación de suelos contaminados por metales pesados, en los últimos años se ha puesto un gran énfasis en la aplicación de bacterias promotoras de crecimiento vegetal - plant growth-promoting bacteria (PGPB) - tanto rizosféricas (PGPR) como endófitas, aisladas de plantas de suelos metalícolas, pues han demostrado un enorme potencial para proteger a las plantas de los efectos tóxicos de los metales pesados y estimular su crecimiento mediante varios mecanismos (Mastretta y cols., 2006; Barzanti y cols., 2007; Chen y cols., 2010; Xinxian y cols., 2011; Zhang y cols., 2011). En este sentido, la biodiversidad microbiana asociada al suelo y/o a las plantas de los entornos mineros también presenta la ventaja de estar adaptada a las condiciones y peculiaridades de este tipo de ambientes.

De este modo, en los Capítulos 5 y 6, se exploró la posibilidad de utilizar bacterias endófitas con características promotoras del crecimiento vegetal (PCV), como una alternativa respetuosa para estimular la biomasa y la tolerancia a los metales pesados de las plantas en procesos de fitoestabilización y fitoextracción. Para ello, se aislaron y caracterizaron bacterias endófitas de 5 especies metalófitas y pseudometalófitas presentes en la mina de Lanestosa (*Festuca rubra*, *Noccaea caerulescens*, *Rumex acetosa*, *Betula alba*, *Salix atrocinerea*), descritas en el Capítulo 2 (Procedimientos Generales). Aunque la inoculación de endófitas no tuvo efecto sobre el crecimiento de las plantas en ninguno de los dos experimentos, en las plantas de *N. caerulescens* (Capítulo 5) se observó un aumento de los niveles de carotenos y clorofilas de hasta un 36 y un 27%, respectivamente, así como una disminución del 24% en los niveles de tocoferol, con respecto a las no-inoculadas. Respecto al proceso fitoestabilizador (Capítulo 6), en las plantas de *F. rubra* inoculadas con la cepa bacteriana endófita, se observaron asimismo niveles mucho más elevados de carotenos, clorofilas y Fv/Fm (39, 65 y 37%, respectivamente) en ambas variedades, a pesar de no mostrar efectos sobre la biomasa. Teniendo en cuenta que los niveles elevados de tocoferol, junto con un bajo contenido en clorofilas, pueden estar relacionados con el daño oxidativo provocado por la toxicidad de metales pesados (Artetxe y cols., 2002), nuestros resultados parecen indicar que la inoculación de endófitas tiene un efecto positivo en el estado fisiológico de las plantas. Esta reducción en el estrés de la planta probablemente se deba a una reducción de los niveles de etileno como consecuencia de la actividad ACC deaminasa (Glick, 2014).

Respecto al efecto de la inoculación de cepas bacterianas endófitas sobre la extracción o estabilización de los metales pesados, aunque diversos autores sostienen que las endófitas con potencial PCV pueden influir en la biodisponibilidad de los metales pesados en el suelo (Mastretta y cols., 2009; Rajkumar y cols., 2009; Chen y cols., 2010), en nuestros experimentos (Capítulos 5 y 6) el efecto de la inoculación de endófitas sobre la fracción biodisponible de metales pesados en el suelo fue demasiado pequeño como para reflejarse en cambios sustanciales en la acumulación (en *N. caerulescens* o *R. acetosa*) o exclusión (en *F. rubra*) de metales pesados por parte de las plantas. Por otro lado, en el ensayo fitoextractor, se observó un aumento en la translocación de Zn en *N. caerulescens* (cuando crece de forma individual); por su parte, en el ensayo fitoestabilizador, se detectó una disminución de la concentración de metales pesados en *F. rubra*, tanto en raíz como parte aérea (solo en ausencia de

enmienda). Esto puede indicar, en el ensayo de fitoextracción, que las bacterias endófitas son capaces de reducir la toxicidad de los metales pesados en las plantas, a la vez que influir en su translocación de la raíz a la parte aérea, posiblemente debido a la producción de sideróforos (Mastretta y cols., 2009; Weyens y cols., 2009); y en el ensayo de fitoestabilización, que las bacterias endófitas también pueden inmovilizar metales pesados en el suelo, reduciendo su biodisponibilidad y dificultando su entrada en la planta, mediante la liberación de fosfato (gracias a su capacidad de solubilizar fosfato inorgánico) (Park y cols., 2011).

Hay que señalar que, en el ensayo de fitoestabilización con *F. rubra*, el efecto positivo de la inoculación con bacterias endófitas sobre (i) el estado fisiológico de las plantas, (ii) la concentración de metales pesados en planta y (iii) la biodisponibilidad de metales pesados en suelo, únicamente se observó en los tiestos en los que no se aplicó enmienda orgánica. Esto demuestra que el efecto de la enmienda enmascaraba cualquier posible efecto del tratamiento con bacterias endófitas. En cualquier caso, los resultados indican que las bacterias endófitas con potencial PCV, aisladas de metalófitas nativas de emplazamientos mineros, pueden tener la capacidad de proporcionar resistencia a los metales pesados y provocar cambios fisiológicos que modulen el crecimiento y desarrollo de las plantas, dotándoles de adaptación para superar los problemas asociados con el crecimiento en suelos mineros, como son la deficiencia de nutrientes y la toxicidad de metales pesados.

Sin embargo, la mayoría de estudios que han demostrado el potencial de las PGPB en procesos de fitoestabilización y fitoextracción han sido efectuados a escala microcosmos, bajo condiciones controladas, y aún hoy en día hay pocos estudios que demuestren la eficacia de su aplicación en campo. Así pues, en el Capítulo 6, de forma paralela al experimento en tiestos, se aplicó un consorcio de bacterias endófitas en un ensayo de fitorremediación en campo en la mina de Lanestosa, y se evaluó su efecto en términos de biomasa de la vegetación nativa (separada en especies exclusoras y acumuladoras) y estabilización de metales pesados en suelo. En este estudio, no se observó que hubiera un efecto en el crecimiento de las plantas, así como en la concentración total o biodisponible de metales pesados en el suelo. Sin embargo, se detectó una reducción en la concentración de Cd y Zn en los tejidos de las especies exclusoras. Estos resultados coinciden con los obtenidos en el ensayo de fitoestabilización a escala microcosmos, indicando el ya mencionado posible efecto

positivo de las bacterias sobre las plantas exclusoras a la hora de dificultar la entrada de metales pesados en la planta.

A pesar del beneficio reportado por las PGPB, la aplicación de enmienda orgánica fue el tratamiento que mayor efecto tuvo, tanto sobre el crecimiento y estado fisiológico de las plantas como sobre la reducción de la biodisponibilidad de metales pesados en suelo y su posterior entrada en las plantas. El uso de enmiendas orgánicas para procesos de fitoestabilización tiene, además, una doble función medioambiental: reducir el riesgo de toxicidad de los metales pesados y reducir el volumen de residuos orgánicos producidos mediante su reutilización (Alvarenga y cols., 2009a; 2009b; Epelde y cols., 2009a). Así pues, se optó por profundizar en el uso de las enmiendas orgánicas para procesos de fitorremediación con el objetivo de abordar las siguientes cuestiones (i) estudiar el efecto a medio-largo plazo de las enmiendas orgánicas (por una posible inversión del efecto estabilizador a consecuencia de la descomposición de la materia orgánica con el tiempo), (ii) evaluar la eficiencia de las enmiendas orgánicas en estudios de fitoestabilización en campo (fuera de las condiciones controladas de microcosmos en una cámara de crecimiento), a escala piloto como paso previo a su aplicación a gran escala, y (iii) buscar la revalorización de los residuos orgánicos mediante su uso en procesos fitorremediadores.

En el Capítulo 7, se realizó un ensayo de quimioestabilización en campo en el que se estudió el efecto de distintas concentraciones de lodo de papelera tratado con cal, como enmienda orgánica, para estimular la revegetación de un suelo minero en la mina de Lanestosa durante un periodo de 6 meses. Como ya se ha comentado anteriormente, la incorporación de enmiendas (orgánicas e inorgánicas) en suelos contaminados por metales pesados, seguida de un periodo de estabilización química, consigue reducir la biodisponibilidad de metales pesados y mejorar la calidad del suelo mediante el incremento del pH, el contenido en materia orgánica y nutrientes, y la capacidad de retención hídrica en el suelo (Alvarenga y cols., 2009a; 2009b). En este sentido, la aplicación del lodo indujo, al cabo de un mes, un incremento del pH, resultando en una reducción en la biodisponibilidad de metales pesados en suelo. Sin embargo, al cabo de 6 meses, los valores de pH volvieron a sus valores iniciales, con el consiguiente aumento en la biodisponibilidad y movilidad de metales pesados, a excepción de las concentraciones de Cd extractable en $CaCl_2$ que se mantuvieron por debajo de los valores iniciales. Estas fluctuaciones no se reflejaron en los valores netos de contenido de metales pesados en planta, por lo que el efecto transitorio de la enmienda no pareció

afectar a la incorporación de metales pesados en planta. Esto puede deberse a la dilución de la concentración de metales pesados en los tejidos de las plantas, provocada por el aumento de la biomasa que se observa con los tratamientos con lodo.

8.2 Indicadores y monitorización de la salud del suelo

En última instancia, la finalidad de un proceso remediador no es eliminar los contaminantes del suelo, o reducir su peligrosidad, sino recuperar la salud del suelo (Epelde y cols., 2009a). Según Doran y Parkin (1996), la salud del suelo se puede definir como ”la capacidad de un suelo de desempeñar sus funciones”. Atendiendo a esta definición, aunque las propiedades físico-químicas (*e.g.*, concentración de metales pesados biodisponibles, pH, etc.) pueden dar una aproximación de la toxicidad potencial de los metales pesados presentes en el suelo, estos parámetros por sí mismos no son suficientes para estimar correctamente el impacto de los metales pesados sobre la salud del suelo y/o para evaluar la efectividad de un proceso de fitorremediación, al menos en términos de la funcionalidad del suelo.

En este sentido, las propiedades microbianas de actividad, biomasa y diversidad han sido señaladas como indicadores idóneos de la salud del suelo y, por tanto, de la funcionalidad del ecosistema edáfico (Epelde y cols., 2008b; 2009c; Gómez-Sagasti y cols., 2012). Para ello, es fundamental disponer de una batería de indicadores microbianos de la salud del suelo que puedan aportar información, tanto del impacto de perturbaciones sobre la funcionalidad y sostenibilidad del suelo como de la recuperación de dicha funcionalidad en procesos de fitorremediación de suelos contaminados. De este modo, el Capítulo 4 se centró en el análisis de parámetros microbianos para evaluar el impacto en el suelo de la contaminación reiterada por metales pesados y valorar su sensibilidad y rapidez de respuesta; y en los Capítulos 5-7, estos parámetros se utilizaron como indicadores de la salud del suelo para valorar la efectividad de distintos procesos fitorremediadores.

A la hora de estudiar el impacto de la contaminación por metales pesados en un suelo, según van Bruggen y Semenov (2000), es altamente recomendable monitorizar la respuesta de los indicadores a distintos intervalos tras la aplicación de una fuente de estrés, para determinar el alcance del impacto y su evolución a lo largo del tiempo. De esta manera, en el Capítulo 4, se monitorizó la respuesta de varios parámetros microbianos, a distintos intervalos después de cada episodio contaminante, con el objetivo de evaluar la evolución de dichos parámetros a lo largo del tiempo en casos de

contaminación reiterada por metales pesados. Así mismo, este análisis temporal aportó información sobre qué parámetros pueden ser más sensibles y rápidos ante cambios (perturbaciones y/o recuperación) en la funcionalidad y sostenibilidad del suelo frente a episodios contaminantes.

Los resultados mostraron que la contaminación repetida de metales pesados, especialmente con Zn y la mezcla de metales pesados, y el consiguiente incremento de la concentración de metales pesados biodisponibles, tuvo un evidente efecto adverso sobre los parámetros microbianos del suelo. Después de todo, los metales pesados en su forma extractable son responsables de su toxicidad y, por ende, los efectos inhibitorios sobre los parámetros microbianos del suelo, incluyendo las actividades enzimáticas (Rusk y cols., 2004).

No obstante, respecto a las propiedades microbianas de actividad (actividades enzimáticas), se observaron claras diferencias en cuanto a la sensibilidad y velocidad de respuesta de unos parámetros a otros ante la presencia de metales pesados. La contaminación con Zn, Cu y mezcla de metales redujeron la actividad fosfatasa ácida ya después del primer episodio contaminante; sin embargo, el Cd y Pb no parecieron tener ningún efecto sobre este parámetro. Por otro lado, la respuesta de la actividad β-glucosidasa al impacto de los metales pesados (únicamente con Zn y la mezcla de metales pesados) fue más lenta, mostrándose a partir del segundo episodio contaminante, mientras que la tasa de nitrógeno potencialmente mineralizable (N_{min}) apenas se vio afectada a lo largo del experimento con ningún metal pesado. Esto indica que la misma actividad enzimática puede responder de forma diferente a metales pesados diferentes y, a su vez, la respuesta de distintas enzimas al mismo metal pesado también puede variar (Zhang y cols., 2010a). Respecto a las propiedades microbianas de biomasa, los índices de abundancia de las comunidades microbianas del suelo, y especialmente de la comunidad fúngica, fueron los más severamente afectados por la mayoría de metales pesados, demostrando ser los más sensibles frente a la contaminación reiterada aquí estudiada.

Independientemente de la dirección de la respuesta (positiva *vs.* negativa), se concluyó que efectivamente los parámetros microbianos son válidos bioindicadores del efecto de los metales pesados sobre la salud del suelo y, por tanto, pueden ser también utilizados como herramientas para evaluar la recuperación de la salud del suelo en procesos fitorremediadores como los realizados en los Capítulos 5-7.

En el Capítulo 5, los indicadores microbianos del suelo corroboraron el efecto positivo que la combinación de *N. caerulescens* y *R. acetosa* había tenido sobre la fitoextracción de metales pesados del suelo: la coexistencia de ambas plantas estimuló el aumento de la biomasa microbiana (reflejada en un incremento de valores de RIS y abundancia de genes bacterianos), así como cambios en su composición (perfiles ARISA), que resultaron en un aumento de la diversidad funcional microbiana del suelo (perfiles fisiológicos a nivel de comunidad, CLPP). Es probable que estos cambios en el crecimiento y diversidad de las comunidades microbianas se deban a cambios en la cantidad y calidad de los exudados de raíz como consecuencia de una mayor diversidad de especies de planta (Yang y cols., 2007; Lucisine y cols., 2014). En cambio, respecto a los indicadores de actividad, en consonancia con lo indicado por Epelde y cols. (2012b), los valores de actividades enzimáticas demostraron estar negativamente correlacionados con la coexistencia de varias especies de plantas.

En el experimento de fitoestabilización (Capítulo 6), el efecto de la enmienda orgánica y, en menor medida, el efecto del uso de la variedad nativa de *F. rubra* Lanestosa demostraron estar relacionados con una estimulación general de las propiedades microbianas, a consecuencia de la reducción de la biodisponibilidad de metales pesados, el aporte de nutrientes y materia orgánica fácilmente degradable, etc. (Alvarenga y cols., 2009a; 2009b; Epelde y cols., 2010b; Bolan y cols., 2011). La respuesta de las actividades enzimáticas a los tratamientos fue muy variable, indicando que de la misma manera que la exposición a metales pesados puede incrementar o disminuir los valores de actividades enzimáticas del suelo dependiendo de la actividad enzimática específica, del metal pesado y de las propiedades del suelo (Lamb y cols., 2009; Zhang y cols., 2010a), las actividades enzimáticas pueden responder de manera diferente al efecto de un mismo proceso fitorremediador.

Respecto al efecto de las bacterias con potencial PCV, la inoculación de bacterias endófitas estimuló la biomasa y la diversidad estructural microbianas en el ensayo de fitoextracción con *N. caerulescens* y *R. acetosa*. Este crecimiento y proliferación de las comunidades microbianas puede deberse a la liberación de azúcares y aminoácidos a la rizosfera (Epelde y cols., 2010b), probablemente como consecuencia de los cambios fisiológicos inducidos en las plantas por la inoculación de las bacterias endófitas. Por el contrario, en el ensayo de fitoestabilización con *F. rubra* (Capítulo 6) no se observó ningún efecto sobre las propiedades microbianas de biomasa o diversidad con la inoculación de bacterias endófitas; aunque sí que se encontraron en los

tratamientos inoculados valores más altos de ciertas actividades enzimáticas (ureasa, deshidrogenasa y FDA). Este efecto tuvo lugar únicamente en los tratamientos sin enmienda orgánica e independiente de la variedad de *F. rubra* empleada, lo que vuelve a indicar que (i) el efecto de la enmienda enmascara el efecto de otros tratamientos, especialmente el de la inoculación de bacterias endófitas, y (ii) las bacterias endófitas no parecen mostrar especificidad con la variedad de planta. Después de todo, bacterias endófitas aisladas de plantas han demostrado efectos positivos en otras especies de plantas, incluso siendo de grupos botánicos distintos (Sessitsch y cols., 2013).

Además, los resultados de la inoculación de bacterias endófitas en ambos experimentos demuestran que el efecto de dichas bacterias sobre el estado fisiológico de las plantas, aunque no repercuta en un incremento de la biomasa vegetal, tiene en última instancia un efecto positivo sobre la salud del suelo, reflejado en una mejora de las propiedades microbianas.

En el ensayo de quimioestabilización en campo (Capítulo 7), se observó que el momento de muestreo tenía una gran influencia sobre los parámetros microbianos, explicando el 83% de la variación total. Esto explicaría que el efecto de los tratamientos con lodo no se mantuviera hasta el final del experimento (6 meses), tal y como sucedió con los valores de pH y metales pesados biodisponibles (excepto Cd) en suelo. Por contra, el efecto pasajero de la enmienda orgánica no pareció reflejarse en los parámetros microbianos. Notablemente, la adición de lodo tuvo un efecto más pronunciado en las propiedades microbianas de actividad, debido a la reducción de Cd biodisponible y el aporte de materia orgánica fácilmente biodegradable resultado de la incorporación de la enmienda. No obstante, no hubo un efecto significativo en la biomasa (carbono de la biomasa microbiana) o en la composición de las comunidades microbianas (PCR-DGGE) del suelo, mientras que sí se observaron diferencias en la utilización de sustratos de carbono, de acuerdo a los resultados obtenidos con los perfiles CLPP (Eco-placas Biolog^{TM}). Esto indica que la adición de lodo pudo estimular la fracción de células activas, aumentando la actividad y diversidad funcional microbianas, mientras que el "pool" de células no-activas presentes en el suelo contribuyó a que no existieran diferencias en los valores de biomasa y composición de las comunidades microbianas. Estas diferencias en cuanto a la diversidad estructural y funcional se deben a que, mientras los perfiles CLPP reflejan el potencial de la porción cultivable heterótrofa de las comunidades bacterians para utilizar fuentes de carbono

(Hitzl y cols., 1997), la técnica PCR-DGGE proporciona un perfil genético de las especies microbianas (*e.g.*, bacterianas) dominantes (Marzorati y cols., 2008).

En cualquier caso, a pesar del evidente efecto positivo de las enmiendas empleadas en los experimentos de fitoestabilización de los Capítulos 6 y 7, el uso de estos métodos químicos para la remediación de suelos parece presentar restricciones, tanto por la falta de permanencia de los efectos observados como por la necesidad de realizar inspecciones regulares para monitorizar su evolución (Tordoff y cols., 2000). Además, la fitoestabilización no elimina los metales pesados del suelo sino que los estabiliza, inmovilizándolos y controlando su potencial toxicidad. Con el objetivo de garantizar que el efecto inmovilizador de la enmienda, y de otros métodos fitoestabilizadores, no es revertido con el tiempo, es altamente recomendable implementar programas de monitorización a largo plazo para comprobar que el riesgo tóxico de los metales pesados se mantiene controlado.

Estos programas de monitorización, en cambio, se encuentran con una serie de dificultades a largo plazo, tales como cambios en los métodos y en las técnicas, nueva legislación, cambios de escenarios, aparición de distintos enfoques y teorías, etc., que inevitablemente irán surgiendo con el tiempo. Por ejemplo, la determinación de la diversidad microbiana, un indicador vital a la hora de monitorizar la salud del suelo, está viviendo en los últimos años avances de gran envergadura a la luz del desarrollo de las tecnologías de secuenciación masiva. Para compensar esta situación, propusimos incorporar el paradigma de la gestión adaptativa, el cual permite a los programas de monitorización evolucionar continuamente en función de los cambios y la información emergente (Epelde y cols., 2014a).

En este sentido, aunque han demostrado ser buenos indicadores de la salud del suelo, los parámetros microbianos resultan ser muy dependientes de su contexto (tipo de suelo, contaminante, etc.), así como variables en el tiempo. Esto supone una dificultad a la hora de interpretar los resultados, especialmente en programas de monitorización a largo plazo, que muchas veces acaba dificultando el entendimiento y la comunicación entre la comunidad científica y el ámbito político-legislador-gestor. Por ello, planteamos el agrupamiento de los parámetros microbianos del suelo en categorías de un nivel superior, tales como *atributos ecológicos* o *servicios de los ecosistemas*, con la finalidad de facilitar la interpretación del diagnóstico y proporcionar a los programas de monitorización de procesos de fitorremediación la estabilidad necesaria frente a

cambios en técnicas, métodos, intereses, etc., que inevitablemente surgen a largo plazo (Garbisu y cols., 2011; Epelde y cols., 2014a).

En los Capítulos 5 y 6, los parámetros del suelo, tanto físico-químicos como biológicos, se agruparon en una serie de servicios de los ecosistemas: *biodiversidad*, *reciclaje de nutrientes*, *almacenamiento de carbono*, *regulación del flujo de agua*, *purificación del agua*, *control de la contaminación*, *control de plagas* y *mantenimiento de la fertilidad*.

Bajo este prisma, los altos valores en *control de la contaminación* y *purificación del agua* volvieron a corroborar la superioridad de *N. caerulescens* para la fitoextracción de metales pesados del suelo, especialmente en combinación con *R. acetosa* (Capítulo 5). Así mismo, la mencionada correlación negativa entre la combinación de ambas especies acumuladoras y las propiedades microbianas de actividad se reflejó en los valores bajos de *reciclaje de nutrientes* (mientras que estas especies de forma individual aumentaban dichos valores). Por el contrario, el aumento en la complejidad estructural y diversidad funcional de las comunidades microbianas del suelo con la coexistencia de *N. caerulescens* y *R. acetosa* se evidenció en un aumento de la *biodiversidad*. Respecto al ensayo de fitoestabilización (Capítulo 6), la mejora de los valores de *control de la contaminación*, *purificación del agua*, *reciclaje de nutrientes* y *almacenaje de carbono* demostraron la eficiencia de la variedad Lanestosa de *F. rubra*, así como de la enmienda orgánica, para recuperar la funcionalidad del suelo en términos de servicios de los ecosistemas, en procesos de fitoestabilización de suelos contaminados con metales pesados. Además, el efecto beneficioso de la enmienda orgánica sobre la estabilización de metales pesados, así como la incorporación de nutrientes y materia orgánica fácilmente biodegradable al suelo (Alvarenga y cols., 2008; 2009a), también se tradujo en una mejora del *mantenimiento de la fertilidad*. En cambio, la adición de purín de vaca tuvo un efecto adverso sobre las comunidades microbianas disminuyendo los valores *de biodiversidad*. El aporte de materia orgánica puede haber propiciado la reproducción de unos determinados grupos microbianos sobre otros, resultando en una pérdida de diversidad microbiana (Tian y cols., 2015).

El efecto de la inoculación de bacterias endófitas, tanto en el ensayo de fitoextracción como en el de fitoestabilización, pareció tener un efecto en los servicios de los ecosistemas relacionados con la actividad microbiana: *reciclaje de nutrientes.* Es posible que la inoculación de las plantas haya inducido cambios a nivel fisiológico

(reflejados en los parámetros fisiológicos de planta) que hayan estimulado la secreción de exudados en la raíz con la consiguiente proliferación microbiana. En el ensayo de fitoestabilización con *F. rubra*, además, la inoculación incrementó los valores de *biodiversidad* y *control de plagas*, siendo este efecto observable solo en tiestos sin purín de vaca, lo que vuelve a demostrar que la enmienda orgánica enmascaraba el efecto de las bacterias endófitas.

Por otro lado, en el Capítulo 7 los parámetros microbianos de suelo se agruparon en una serie de atributos de relevancia ecológica: *vigor*, *organización*, *estabilidad*, *redundancia* y *supresividad*. El efecto de la aplicación de lodo sobre los atributos solamente se observó en el *vigor*, el cual está relacionado con la actividad, el metabolismo y la producción primaria de un ecosistema. Esto viene a recalcar lo anteriormente mencionado acerca del efecto más pronunciado de la enmienda sobre los parámetros microbianos de actividad, como consecuencia de la incorporación de nutrientes en el suelo y la reducción en la biodisponibilidad de metales pesados. También se observó un efecto sobre la *estabilidad* del suelo, la cual está calculada a partir de estimaciones de la *resistencia* y *resiliencia* del suelo. Sin embargo, estas estimaciones variaban en función del momento de muestreo y de los parámetros empleados para su valoración (actividad deshidrogenasa y tasa potencial de nitrificación). Por otro lado, la *estabilidad* del suelo parece estar fuertemente vinculada a la biodiversidad, ya que, si bien no hay aún una comprensión total de los mecanismos subyacentes, según Loreau y de Mazancourt (2013), la biodiversidad incrementa la estabilidad de los procesos del ecosistema a lo largo del tiempo. En este sentido, la ausencia de cambios en la composición de las comunidades microbianas (perfiles PCR-DGGE), así como la variabilidad en los parámetros utilizados para calcular la estabilidad del suelo, parecen explicar que el efecto de lodo sobre este atributo sea transitorio y se disipe con el tiempo. Además, el efecto de la enmienda sobre la diversidad funcional microbiana, reflejada en la riqueza de utilización de sustratos de carbono (obtenidos con los perfiles CLPP), parece contribuir más sobre la mejora de atributos basados en actividad, como el *vigor*, que en otros que dependen en mayor medida de las relaciones entre los componentes de las comunidades microbianas, como la *estabilidad* o la *organización* del suelo.

Como se puede observar en los distintos Capítulos de este trabajo, los parámetros microbianos de actividad, biomasa y diversidad han demostrado ser idóneos indicadores de la salud del suelo, aportando información complementaria a la obtenida

con los parámetros físico-químicos, puesto que proporcionan estimaciones de la funcionalidad del ecosistema edáfico. La sensibilidad, rapidez en la respuesta y relevancia ecológica de los parámetros microbianos han permitido evaluar el impacto y la evolución de la contaminación reiterada por metales pesados sobre la salud del suelo. Análogamente, estas mismas propiedades aportan información que integra diversos factores ambientales, lo cual es de gran importancia a la hora de evaluar la eficiencia de procesos fitorremediadores de suelos contaminados como los realizados en los Capítulos 5-7. Prueba de ello es que los incrementos en la biomasa observados en las plantas en los distintos experimentos de fitorremediación (variedad Lanestosa de *F. rubra* *vs.* variedad comercial, combinación de *N. caerulescens* y *R. acetosa* *vs.* monocultivos, enmienda *vs.* no enmienda) han ido asociados a una estimulación de las propiedades microbianas, demostrando así mismo lo descrito por Epelde y cols. (2010b): la biomasa de las plantas puede tener un efecto más fuerte que la diversidad vegetal sobre los parámetros microbianos.

Las conclusiones acerca del efecto de los tratamientos en los ensayos de fitoestabilización y fitoextracción, obtenidas a partir de la agrupación de los parámetros del suelo en servicios de los ecosistemas coinciden con las obtenidas a partir de la valoración de los indicadores de forma individual. En cambio, la estimación de los atributos ecológicos, en el ensayo de quimioestabilización en campo, proporcionó una interpretación del efecto de los tratamientos que no coincidía totalmente con la obtenida con los indicadores por separado. Hay que señalar que los atributos se calcularon únicamente a partir de parámetros microbianos, no incluyendo parámetros físico-químicos de gran relevancia (como las concentraciones de metales pesados en el suelo), mientras que para los servicios de los ecosistemas se tuvieron en cuenta tanto propiedades biológicas del suelo como físico-químicas. En este sentido, la valoración de los atributos ecológicos posee un componente basado únicamente en propiedades microbianas del suelo, profundizando más en la comprensión de la actividad, composición e interrelaciones de las comunidades microbianas. Esto explica que la observación de los atributos ecológicos aportara una interpretación adicional del efecto de los tratamientos. En cualquier caso, en la medida de lo posible, siempre es recomendable llevar a cabo una interpretación tanto a nivel de indicador, como a nivel de categoría de nivel superior.

Respecto a la elección de categoría de nivel superior (atributos ecológicos *vs.* servicios ecosistémicos), también se recomienda la utilización de ambas para una

interpretación complementaria de los resultados. De esta manera, se obtiene una estimación, por un lado, bajo una óptica ambiental (atributos ecológicos), y por otro, en clave más antropocéntrica, valorando mayormente la obtención de beneficios para el ser humano (servicios de los ecosistemas). Además, la evaluación de la salud del suelo mediante la utilización de atributos ecológicos o servicios de los ecosistemas facilita la comprensión y comunicación entre el sector científico y el gestor-legislador, puesto que términos como "vigor" o "biodiversidad" siempre serán más fácilmente interpretados y asimilados por el ámbito no-científico que términos más técnicos como, por ejemplo, "respiración basal" o "actividad fosfatasa ácida".

8.3 ¿Y ahora qué?

La fitorremediación tiene un futuro prometedor, presentándose como una fitotecnología ambientalmente respetuosa y de bajo coste para la recuperación de suelos contaminados con metales pesados. Sin embargo, a pesar del enorme avance de las últimas décadas, la fitorremediación sigue encontrándose con una serie de dificultades y limitaciones que entorpecen su desarrollo. El principal obstáculo está relacionado con los largos plazos de tiempo requeridos para alcanzar y/o asegurar un nivel de recuperación del suelo que sea aceptable. Por otra parte, estos largos periodos de tiempo implican la necesidad de implementación de programas de monitorización con el objetivo de garantizar, por un lado, un control del riesgo de toxicidad de los contaminantes y, por otro, que la mejora en la salud del suelo permanezca como tal.

Pero la monitorización a largo plazo también se enfrenta a una serie de retos que inevitablemente aparecen con el tiempo, tales como la aparición de nuevas técnicas y métodos, distintos enfoques e intereses, nuevas teorías científicas, nuevos escenarios debido a distintas amenazas ambientales, situaciones políticas y económicas oscilantes, nueva legislación, y otras circunstancias asociadas a cada momento histórico. En este sentido, la incorporación del paradigma de la gestión adaptativa, por el cual los programas de monitorización evolucionan y son revisados continuamente, es altamente recomendable. De este modo, por ejemplo, los aspectos químicos, toxicológicos y ecológicos, relacionados con la contaminación por metales pesados, tenidos en cuenta en el diseño del programa de monitorización pueden ser revisados y actualizados periódicamente por un panel de expertos de distintos ámbitos (científico, político-legislador, gestor, etc.). La revisión de los distintos aspectos que componen los programas de monitorización proporciona, de este modo, la estabilidad necesaria frente

a cambios en técnicas, métodos, intereses, etc., que inevitablemente surgirán a lo largo del tiempo.

Así mismo, la participación de equipos de investigación multidisciplinares, que realicen una valoración de los logros y fracasos obtenidos en ensayos de fitorremediación, particularmente en campo, es imperante para determinar la sostenibilidad ecológica, social y económica de las distintas fitotecnologías, así como para demostrar su eficiencia, con el propósito de promover su implementación. Además, debe darse un cambio de práctica hacia una "fitogestion" sostenible de los emplazamientos contaminados con metales pesados, en los que las fitotecnologías empleadas, no solo tengan el objetivo de reducir o contener la toxicidad de los contaminantes, sino que también se utilicen como parte de una gestión integrada que tenga en consideración la obtención de una serie de beneficios económicos, sociales y ambientales más amplios.

Estos cambios de enfoque y práctica (agrupación de indicadores del suelo en categorías superiores, gestión-monitorización adaptativa, participación de equipos multidisciplinares, fitogestión, etc.) en la implementación y seguimiento de distintas fitotecnologías, además de facilitar la evaluación de los procesos de fitorremediación, buscan revalorizar los beneficios obtenidos del ecosistema edáfico. La consideración de estos beneficios de carácter más amplio (*e.g.*, ecológicos, económicos, sociales y culturales), pero de enorme valor para la humanidad, tiene el potencial de cambiar esa percepción de que la fitorremediación no es una técnica viable, así como promover la aceptación y participación de gestores y legisladores. De este modo, la fitorremediación puede ser implementada en emplazamientos marginales de bajo valor, o incluso en suelos que han sido sometidos a procesos severos de remediación mediante técnicas físico-químicas destructoras de la estructura y funcionalidad del suelo. En este sentido, es importante recalcar que la fitorremediación es una tecnología que con relativa rapidez puede recuperar la salud de un suelo contaminado.

Por último, hay que enfatizar, una vez más, que el suelo es un recurso no renovable del cual el ser humano obtiene infinidad de beneficios, y cuya integridad es esencial para el mantenimiento de los ecosistemas terrestres. La recuperación de los suelos degradados, como consecuencia de la contaminación por metales pesados, es vital para la sostenibilidad del sistema edáfico y para nuestra propia supervivencia. Por otra parte, teniendo en cuenta la importancia de las comunidades microbianas edáficas en la correcta funcionalidad del ecosistema edáfico, es necesario que una parte

importante de los esfuerzos dedicados a la investigación en este campo vaya orientada a elucidar el rol y la complejidad de dichas comunidades microbianas. Así mismo, no es casualidad que el desarrollo de nuevas y prometedoras fitotecnologías en los últimos años parezca haber otorgado especial significancia al papel de los microorganismos, tanto del suelo como asociados a plantas. Además de reafirmar la importancia de las comunidades microbianas como indicadores imprescindibles de la salud del suelo, estas tecnologías asignan a los microorganismos un papel protagonista en el propio proceso fitorremediador.

Por desgracia, estamos muy lejos de una comprensión total de los roles y mecanismos de estos microorganismos y su interacción con el sistema suelo-planta. En este sentido, la aparición y desarrollo de técnicas "ómicas" moleculares (*e.g.*, metagenómica, metatranscriptómica, metaproteómica y metabolómica), métodos de análisis de imagen, así como de herramientas bioinformáticas capaces de procesar la enorme cantidad de datos resultantes, arrojarán sin duda luz sobre la ecología de las plantas, sus microorganismos asociados y, especialmente, sobre la complejidad de las interacciones contaminante-suelo-planta-microorganismo. Confiamos que una interpretación mecanística de esta información contribuirá enormemente a optimizar los procesos de fitorremediación, facilitando su adaptación a distintos ambientes y futuros escenarios.

09 | CONCLUSIÓN Y TESIS

9. CONCLUSIONES Y TESIS

9.1 Conclusiones

1. La biodisponibilidad de metales pesados en el suelo no estuvo correlacionada con su concentración total. La biodisponibilidad relativa de los metales pesados, ante la contaminación reiterada con uno o varios metales simultáneamente, aumentó con los sucesivos episodios contaminantes.

2. Las propiedades indicadoras de biomasa microbiana, así como la actividad enzimática fosfatasa ácida, fueron los parámetros más sensibles al efecto tóxico de la contaminación reiterada con uno o varios metales pesados simultáneamente.

3. La combinación de *Noccaea caerulescens* y *Rumex acetosa* aumentó el potencial fitoextractor de estas especies en suelos contaminados con metales pesados, y produjo un incremento de la biomasa de las plantas, una reducción de la biodisponibilidad de los metales, y una estimulación de las propiedades microbianas de biomasa y diversidad.

4. El ecotipo Lanestosa de la especie *Festuca rubra*, en comparación con una variedad comercial, presentó una mayor tolerancia y adaptación a los suelos contaminados con metales pesados, mostrando escasos signos de fitotoxicidad y un mayor potencial fitoestabilizador, reflejado éste en una disminución de los metales biodisponibles en el suelo y una mejora de las propiedades microbianas de actividad, biomasa y diversidad.

5. Las especies (pseudo)metalófitas nativas de entornos mineros tienen un gran potencial para la fitorremediación de suelos contaminados con metales pesados.

6. La aplicación de enmiendas orgánicas, concretamente purín de vacuno, en procesos fitoestabilizadores tuvo un efecto positivo sobre el crecimiento de las plantas y la salud del suelo, disminuyendo la biodisponibilidad de metales pesados e incrementando los valores de las propiedades microbianas. No obstante, la adición de la enmienda tuvo un efecto negativo sobre la diversidad de las comunidades microbianas del suelo.

7. La adición de enmiendas orgánicas, concretamente lodo de depuradora tratado con cal, en procesos de quimioestabilización de suelos mineros tuvo un efecto transitorio sobre las propiedades físico-químicas del suelo. Sin embargo, la aplicación de lodo condujo a una mejora en las propiedades microbianas edáficas, especialmente en aquellas relacionadas con la actividad microbiana, así como en el crecimiento de las plantas.

8. La inoculación con bacterias endófitas promotoras del crecimiento vegetal, aisladas de plantas nativas de entornos mineros, no afectó a la biomasa de las plantas empleadas en fitorremediación, pero sí mejoró su estado fisiológico, resultando, en última instancia, en un efecto positivo sobre la salud del suelo reflejado en la mejora de las propiedades microbianas.

9. Las propiedades microbianas del suelo fueron indicadores idóneos tanto del impacto a lo largo del tiempo de la contaminación por metales pesados como de la eficacia de procesos fitorremediadores.

10. La estimación de atributos ecológicos y servicios de los ecosistemas, a partir del agrupamiento de las propiedades del suelo, proporcionó información de gran relevancia ecológica acerca de la funcionalidad del ecosistema edáfico, aportando una interpretación complementaria de cara a evaluar la eficacia de procesos fitorremediadores.

9.2 Tesis

La fitorremediación y la fitogestión son fitotecnologías que aportan mejoras sustanciales en la funcionalidad del suelo, convirtiéndolas así en alternativas que ofrecen, frente a las tradicionales técnicas físico-químicas empleadas en la remediación de suelos contaminados, beneficios añadidos a la propia eliminación del riesgo ocasionado por los metales pesados. En este sentido, las propiedades microbianas con potencial indicador de la salud del suelo se presentan como herramientas valiosas para la evaluación del impacto de la contaminación por metales pesados, así como de la eficacia de procesos fitorremediadores. Así mismo, la estimación de atributos de relevancia ecológica y servicios de los ecosistemas, mediante la agrupación de propiedades microbianas del suelo, se ofrece como una metodología adecuada que aporta información complementaria y facilita la interpretación de los resultados.

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ANEXO

Graphical representation of metal phytoremediation and soil health.