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TESIS DOCTORAL

ASSESSMENT OF PHYTOSTABILIZATION OF PB/ZN MINE SOILS WITH ORGANIC AMENDMENTS AND A NATIVE METALLICOLOUS ECOTYPE OF *FESTUCA RUBRA* L.: IMPLICATIONS FOR AMENDMENT SELECTION AND RECOVERY OF SOIL HEALTH

Memoria presentada por **María Asunción Galende Gullón** para optar al grado de Doctor

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En 1977, la NASA envió al espacio dos naves con el objetivo de explorar el sistema solar y 35 años más tarde las sondas Voyager aún siguen recorriendo la inmensidad espacial. Las sondas se usaron como cápsulas del tiempo y llevaban un mensaje que comunicase la historia de nuestro mundo a posibles formas de vida inteligente extraterrestres. El mensaje, grabado en el Disco de Oro, contiene sonidos e imágenes que reflejan la diversidad de la vida y la cultura en la Tierra: la fauna, la flora, el viento, el agua, la música,… Imágenes bellas y sonidos extraordinarios de un planeta que a día de hoy cambia su aspecto a pasos agigantados. El lanzamiento de estas botellas al océano cósmico transmitiría un mensaje optimista sobre la vida en nuestro planeta.

Me paro a pensar en qué estamos haciendo con nuestro planeta, ¿por qué si nos ofrece todo aquello que necesitamos para vivir, no somos capaces de respetarlo? Ha llegado el momento de optar por un cambio y empezar a respetar nuestro entorno y a nosotros mismos. Aún estamos a tiempo de parar los conflictos y de hacer que la Tierra sea lo que las sondas Voyager dicen que es: un mundo extraordinariamente bello que vive en paz.

A mis padres Fe y Luis
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ABSTRACT

Soil pollution is one the most pressing threats currently affecting ecosystems and human health. Nowadays, phytostabilization is the most cost-effective and environmentally friendly *in-situ* technology for remediation of soils contaminated with high levels of metals, as mine soils. Aided phytostabilization consists of using both plants and organic/inorganic amendments in order to reduce contaminants mobility and bioavailability and recover soil health. In this work, microcosms and field studies are focused on soil health of a Pb/Zn mine soil during phytostabilization process using organic amendments (cow slurry, poultry manure, sheep manure and paper mill sludge mixed with poultry manure) and/or the native metallicolous species *Festuca rubra,* in order to: (i) study soil-amendment interactions responsible for the stabilization-induced changes in soil physicochemical and biological properties, (ii) assess the effectiveness of amendments for phytostabilization on vegetated soils and revegetation of bare soils and (iii) assess the suitability of several chemical and biological indicators (plant and microbial parameters) to monitor the effectiveness of aided phytostabilization in terms of reduction of soil metal bioavailability, improvement of vegetation performance and soil health recovery. Amendment application to the mine soil involved an input of organic matter and nutrients which led to a decrease in soil metal availability. This factors facilitated plant colonization, native plant growth and reduction in metal phytoavailability, as well as stimulated soil microbial activity. Soil pH was a crucial factor conditioning metal mobility and soil toxicity. Besides, microbial populations from the amendments did not modify functional diversity of native microbial communities of mine soil. Cow slurry and paper mill sludge mixed with poultry manure were the most effective treatments in aided phytostabilization processes under field conditions. Poultry manure was the best treatment to stimulate native spontaneous vegetation and to promote plant growth. Plant based test, as root elongation bioassay, is a sensitive, straightforward, cost effective method to assess metal bioavailability and soil ecotoxicity. Other biomarkers of phytotoxicity, as tocopherols, could be also implemented for new bioassays. This work concludes that metallicolous population of *F. rubra*, combined with organic amendments, is an excellent candidate for aided phytostabilization projects. Moreover, simultaneous monitoring of soil physicochemical and microbial parameters and soil ecotoxicity allow the proper evaluation of soil health, as well as the selection of appropiate amendments for the development of a phytostabilization process.

RESUMEN

La contaminación del suelo es una de las principales amenazas para los ecosistemas y la salud humana. Actualmente, desde un punto de vista tanto económico como ambiental, la fitoestabilización es la mejor tecnología para remediar suelos contaminados con elevadas concentraciones de metales como son los suelos mineros. La fitoestabilización asistida consiste en el empleo de plantas y enmiendas orgánicas y/o inorgánicas con el fin de reducir la movilidad y la biodisponibilidad de los contaminantes y recuperar la salud de suelo. En este trabajo se han realizado ensayos en microcosmos y en campo centrándonos en la salud del suelo minero contaminado con Pb y Zn durante un proceso de fitoestabilización empleando enmiendas orgánicas (purines vacunos, gallinaza, estiércol de oveja y lodos de papelera mezclados con gallinaza) y/o la especie metalífera *Festuca rubra* con el objetivo de (i) estudiar las interacciones suelo-enmienda responsables de los cambios inducidos por el proceso de quimioestabilización en las propiedades físicoquímicas y biológicas del suelo, (ii) evaluar la efectividad del proceso de fitoestabilización sobre suelos vegetados y de la revegetación sobre suelos desnudos (iii) valorar la idoneidad de distintos indicadores químicos y biológicos (parámetros microbianos y de la vegetación) para monitorizar la efectividad de la fitoestabilización asistida en términos de reducción de la biodisponibilidad de metales en el suelo, mejora de la vegetación y de la recuperación de la salud del suelo. La aplicación de enmiendas al suelo minero supone una entrada de materia orgánica y nutrientes que conduce a una disminución de la biodisponibilidad de metales, facilitando la colonización de las plantas y el crecimiento de la vegetación nativa, además de estimular la actividad microbiana del suelo. El pH del suelo es un factor crítico que condiciona la movilidad de los metales y la toxicidad del suelo. Las poblaciones microbianas de las enmiendas no modificaron la diversidad funcional de las comunidades microbianas nativas de la mina. Los purines vacunos y los lodos de papelera mezclados con gallinaza son los tratamientos más efectivos en el proceso de fitoestabilización asistida bajo condiciones de campo. La gallinaza fue el tratamiento que más estimuló el crecimiento de la vegetación nativa y la colonización en los suelos desnudos. El bioensayo de elongación radical de lechuga es un test sensible, sencillo y barato para evaluar la biodisponibilidad de metal y la ecotoxicidad del suelo. Los tocoferoles son biomarcadores de exposición a metales con potencial para su implementación en bioensayos de toxicidad. Este trabajo permite concluir que la población metalífera de *F. rubra*, combinada con enmiendas orgánicas, es una excelente candidata para los proyectos de fitoestabilización asistida. Además, la monitorización simultánea de los parámetros fisicoquímicos y microbiológicos del suelo y de su ecotoxicidad permite una evaluación adecuada de la salud del suelo, así como la selección de enmiendas apropiadas para el desarrollo de un proceso fitoestabilizador.

LABURPENA

Lurzoruaren poluzioa ekosistema eta giza osasunerako mehatxu nagusienetarikoa da. Gaur egun, ekonomiaren zein ingurunearen ikuspuntutik, fitoegonkortzea (lagundutako egonkortzea) metal-kontzentrazio altuekin kutsatutako meatze-lurzoruak erremediatzeko teknologiarik onena da. Lagundutako egonkortzea landareen eta medeatzeen (organikoen eta ez-organikoen) erabileran datza, metal kutsakorren mugikortasuna eta bioeskuragarritasuna murriztuz, eta lurzoru-osasuna berreskuratuz. Lan honetan medeatze organikoak (behi-mindak, oilo–zirina, ardi zimaurra, eta oilo–zirinarekin nahasitako papergintza-lohiak) eta *Festuca rubra* metal-mehatze lurzoruko espeziea erabili dira. Fitoegonkortze prozesuaren funtzionamendua aztertzeko mikrokosmosetan eta landa-baldintzetako entseguak egin dira, metalekin poluitutako meatze-lurzoruen osasuna kontuan hartuta. Lan honetan hurrengo hiru helburu aztertu dira: (i) lurzoruaren eta medeatzearen arteko interakzioak ikertzea. Hauek quimioegonkortze prozesuak eragindako lurzoruko ezaugarri fisiko-kimikoen eta biologikoen aldaketen arduradunak dira; (ii) lurzoru landatuetan fitoerremediazioaren eta landarediarik gabeko lurzoruen bir-begetatzearen eraginkortasuna aztertzea, eta (iii) lagunduriko egonkortzearen eraginkortasuna jarraitzeko, adierazle kimiko eta biologiko batzuen (propietate mikrobianoak eta landarediarena) egokitasuna baloratzea eta, honekin batera, lurzoruko metalen bio-eskuragarritasuna, landareen hobekuntza eta lurzoruko osasunaren berreskuratzea analizatzea. Meatze-lurzoruan, lurzoruko mikrobioen jarduera estimulatzeaz gain, medeatze aplikazioak elikagaien eta materia organikoaren sartzea eta metal-bioeskuragarritasunaren jaitsiera dakartza eta horrek landareen kolonizazioa eta landaredi natiboaren hazkundea daramatza. Lurzoruko pH-a, metalen eskuragarritasuna eta ondorioz lurzoruaren toxikotasuna baldintzatzen dituen faktore kritikoa da. Medeatzeetako mikrobioen populazioek ez zuten aldatu meategiko mikrobio-komunitate natiboen aniztasun funtzionala. Landa baldintzetan, behi-mindak eta oilo–zirinarekin nahasitako papergintza-lohiak tratamendurik eraginkorrenak dira lagundutako egonkortze prozesuan. Beste aldetik, landaredi natiboaren hazkundea eta lurzoru biluzietako kolonizazioa gehien estimulatu zituen tratamendua oilo–zirina izan zen. Urazaren sustrai-luzapenaren bioentsegua, metal-bioeskuragarritasuna eta lurzoruko ekotoxikotasuna aztertzeko, sentikorra, erraza eta merkea den proba da. Tokoferolak metalekiko esposizio-adierazleak direnez, toxikotasun-entseguak burutzeko ahalmen handia dute. Funtsean, *F. Rubra* meatze populazioa medeatze organiko batzuekin konbinatuta, hautagai bikaina da landareekin lagundutako egonkortze proiektuetan. Gainera, lurzoruaren ekotoxikotasuna eta ezaugarri fisiko-kimiko zein mikrobiologikoak aldi berean neurtzeak lurzoruaren osasuna ebakuatzea, eta, horrez gain, fitoegonkortze prozesua burutzeko zeintzuk diren medeatze-teknikarik egokienak ezagutzea baimentzen ditu.

INDEX

1. General Introduction

Al olmo viejo, hendido por el rayo
y en su mitad podrido,
con las lluvias de abril y el sol de mayo
algunas hojas verdes le han salido.
¡El olmo centenario en la colina
que lame el Duero! Un musgo amarillento
le mancha la corteza blanquecina
al tronco carcomido y polvoriento.
No será, cual los álamos cantores
que guardan el camino y la ribera,
habitado de pardos ruiseñores.

(Antonio Machado)

1. General Introduction

1.1. Metal-polluted mine soils[1]

Metal-polluted soils, from both natural and anthropogenic origin, are a worldwide environmental problem because of the well-known toxicity of metals. Natural sources of metals include weathering of parent rock material, volcanic outcropping, forest fires, etc. (Singh and Prasad, 2011), while anthropogenic sources can be divided into five main groups (Ross, 1994b; Shen et al., 2002): metalliferous mining and smelting, industrial activities (fuel production, military operations, coal burning, effluents), atmospheric deposition, agricultural practices (application of pesticides and fertilisers), and waste disposal.

Indeed, as a consequence of the industrial revolution, the use and dispersion of metals in the environment during the 20th century reached enormous proportions, thereby becoming an environmental problem of great concern (Nriagu, 1990). On the other hand, there is an increasing demand worldwide for metals as raw materials, leading to a further intensification of mining activity (Furrer et al., 2002).

As a result, the health of our soils is currently at great risk with respect to metal pollution. Soils are a multi-functional resource that provide a range of essential ecosystem goods and services (i.e., the multiple benefits provided by ecosystems to humans), such as organic matter recycling and fertility, regulation of carbon flux and climate control, water cycle regulation, decontamination and bioremediation, pest control, etc. (Turbé et al., 2010). Regrettably, human activities have exceeded the capacity of our soils to withstand pollution and remediate themselves. Therefore, soil remediation and, in general, conservation is currently a priority in many countries.

[1] From 1.1 to 1.5: Galende et al. (2013) published in de Bruijn FJ (Ed.) Molecular Microbial Ecology of the Rhizosphere. John Wiley & Sons, Hoboken, New Jersey, USA. ISBN:978-1-1182-9617-2

1.2. Impact of metals on plants and soil microbial communities

Microorganisms comprise the majority of the biomass and biological diversity of the soil and they are, to a great extent, responsible for providing many of the soil ecosystem services on which human society relies. Furthermore, plant-microorganism interactions in the rhizosphere are essential for plant health and soil fertility (Nwoko, 2010). Rhizosphere microorganisms, including free-living and symbiotic rhizobacteria and mycorrhizal fungi (Karami and Shamsuddin, 2010), are known to (i) stimulate plant growth through the production of growth regulators, (ii) enhance mineral and water uptake (Nwoko, 2010) and (iii) increase plant tolerance to various environmental stresses (Glick, 2004). For their part, plant root exudates provide a source of nutrients for rhizosphere bacteria and fungi (Bowen and Rovira, 1991), resulting in a greater development of microbial populations and associated processes compared to surrounding bulk soils (Hawkes et al., 2007).

Metals and metalloids are potentially harmful to all biota. Cadmium (Cd), lead (Pb), cobalt (Co), copper (Cu), mercury (Hg), nickel (Ni), manganese (Mn) and zinc (Zn) are some of the most frequently found in polluted soils (Alkorta et al., 2010). The ecotoxicological risk of metal pollutants depends on, among other factors, the type of metal and its concentration (total and bioavailable). In any case, both essential (Zn, Co, Cu, Mn, Ni) and non-essential (Pb, Cd, Hg) metals can affect plant and microorganism health.

Due to metal pollution, plants may undergo, for instance, a reduction in photosynthetic activity (Becerril et al., 1989), inhibition of root cell elongation (Bakos et al., 2008) and alteration of key enzymes in various metabolic pathways, leading to chlorosis symptoms and growth inhibition (Arduini et al., 1996). Accordingly, soils polluted with high concentrations of metals (e.g., mine soils) can restrict the growth of all but the most tolerant plants (Wong, 2003), resulting in a sparse plant cover. However, metallophytes (metal tolerant species endemic of polluted sites) and metallicolous populations of pseudometallophytes (plant

species having both metallicolous and non-metallicolous populations) have developed cellular and molecular mechanisms for metal detoxification and tolerance, allowing them to survive in metal-polluted soils. Plants can show different metal tolerance mechanisms (Hall, 2002): (i) reduced uptake or efflux pumping of metals at the plasma membrane level, (ii) metal binding to root cell wall and extracellular root exudates, such as low-molecular-weight organic acids which can complex metals reducing their bioavailability (Wang et al., 2009), (iii) vacuolar compartmentalization of metals, (iv) improved repair mechanisms of plasma membranes, (v) chelation of metals in the cytosol by amino acids, carboxilic acids and peptides such as phytochelatins and metallothioneins, etc.

The presence of high concentrations of bioavailable metals in mine soils exerts a selective pressure on colonizing plants, frequently resulting in a specific and unique community of plant species (Barrutia et al., 2011; Whiting et al., 2004). The preservation of the metallophyte germplasm present in mine soils is of great interest for the study of metal tolerance mechanisms, as well as due to the potential value of those plant species for soil metal phytoremediation (Barrutia et al., 2011). However, sound decisions on the sustainable management of these metalliferous ecosystems are impeded by a lack of ecological understanding of how metal pollutants, ecosystem functions and biological communities interact in the long-term (Ramsey et al., 2005). Moreover, many metallophytes are nowadays endangered because of their restricted and dispersed habitat (Whiting et al., 2004; Pauwels et al., 2008) and/or human threats (alterations in land use, climate change, etc.). An appropriate management of metalliferous sites must ensure the conservation of this unique metallicolous biodiversity (Barrutia et al., 2011; Whiting et al., 2004).

Likewise, metals have been reported to damage cell membranes and DNA structure, alter enzyme specificity and disrupt cellular functions of microorganisms (Bruins et al., 2000). In fact, the presence of toxic metals in soil can negatively interfere with the key roles played by soil microorganisms in the major biogeochemical cycles (Haferburg and Kothe, 2010).

Microorganisms from metalliferous soils are adapted to survive under the high metal concentrations found in mine soils thanks to the development of intracellular and extracellular resistance mechanisms mediated through chromosomal, plasmid or transposon resistance systems (Bruins et al., 2000). Microorganisms exhibit several different metal resistance mechanisms such as: (i) exclusion by permeability barriers through alterations in the cell wall, membrane or envelope, (ii) active transport of the metal away from the cell by efflux systems, (iii) intracellular sequestration by protein binding in the cytoplasm (via metallothioneins and other cysteine-rich proteins), (iv) extracellular sequestration through substrate excretion of molecules which precipitate metals or form complexes with them, thereby preventing metals from entering the cells, (v) enzymatic detoxification to less toxic forms, and (vi) reduction in the vulnerability of essential cellular components to the presence of metals, by means of mutations, production of metal-resistant components, etc. (Bruins et al., 2000). Indeed, metal pollution also exerts a strong pressure on soil microorganisms, resulting in the recruitment of genotypes that provide tolerance to the metals present in the polluted site (Ryan et al., 2005).

Metal-tolerant rhizosphere microorganisms can increase metal mobility and bioavailability (i.e., the fraction of the total metal content of the soil that can interact with a biological target) (Geebelen et al., 2003) to plants through the release of chelating agents, acidification, phosphate solubilisation, redox changes, etc. (Abou-Shanab et al., 2003; Idris et al., 2004; Ma et al., 2009). Alternatively, rhizosphere microorganisms can reduce metal phytotoxicity through, for instance, the production of siderophores, which apart from increasing the supply of iron to the plants, alleviate metal-induced phytotoxicity by siderophore-metal complexes formation (Dimkpa et al., 2008; Tripathi et al., 2005).

With respect to the interactions between plant and rhizosphere microbial communities in mining soils, we found soil microbial properties to have a stronger effect on plant biomass rather than the other way round (Epelde et al., 2010).

This was explained by the extremely harsh conditions present in the mine soil, making soil microbial activity of vital importance for plant development. Certainly, positive effects of soil microorganisms on plant productivity are most common in nutrient-poor ecosystems, such as mine soils, where they enhance the supply of growth limiting nutrients (nitrogen, phosphorus, etc.) to plants (Van der Heijden et al., 2008).

1.3. Strategies for metal remediation

Traditional physicochemical methods of soil metal remediation such as excavation, isolation, chemical fixation, *in-situ* solidification, washing, etc. are expensive and, often, have a strong adverse impact on the integrity of the soil ecosystem and the vital ecosystem services provided by it.

In the last decades, environmentally-friendly, cost-effective biological strategies of soil metal remediation have been developed. Among these strategies, phytoremediation, or the use of green plants, to remove pollutants from contaminated sites or to render them harmless (Cunningham and Berti, 1993; Raskin et al., 1994), appears to be highly promising.

Within the phytoremediation field, plants can be used to extract (phytoextraction, rhizofiltration), degrade (phytodegradation), volatilize (phytovolatilization) or stabilize (phytostabilization) pollutants. Furthermore, phytoremediation effectiveness is sometimes enhanced by the use of mycorrhizas, chelating agents, organic and inorganic amendments, plant growth-promoting bacteria, or genetic engineering approaches (Pilon-Smits, 2005). Depending on the specific site to be remediated, phytoremediation is also confronted with several important challenges such as, for instance, the deficiency of essential nutrients and soil structure characteristic of mining areas or adverse climatic conditions, which may impede plant growth. There are also constraints that can limit the application of phytoremediation strategies, such as the long

periods of time required for soil pollutant removal and its lack of applicability when pollutants are located in the deep soil layers not penetrated by roots (Karami and Shamsuddin, 2010).

When the soil is polluted with high concentrations of metals, phytoextraction, or the use of plants (usually, hyperaccumulators) to transport and concentrate metals from the soil into their harvestable parts, is not a feasible option due to the long time required. In this case, phytostabilization, which involves the establishment of a plant cover on the surface of metal polluted soils in an attempt to reduce metal mobility and bioavailability (Bennett et al., 2003; Jing et al., 2007), is usually the option of choice. The most important processes involved in this reduction of metal mobility and bioavailability are: (i) uptake and sequestration of metals in the root system, (ii) alteration of soil properties, such as pH, organic matter content and redox potential, and (iii) production of root exudates which affect metal speciation and immobilization (Bolan et al., 2011; Wang et al., 2009). Most importantly, during phytostabilization processes, plants, especially excluder species act as a physical barrier, thereby preventing direct contact of animals and humans with the polluted soil and reducing wind erosion and dust dispersion, and minimizing off-site soil pollution (Vangronsveld et al., 2009). On the other hand, the reduction in metal bioavailability prevents leaching and runoff, thus protecting ground and surface waters (Raskin and Ensley, 2000). Once the metals are stabilized, they become less available for plants, diminishing their entrance into the food chain (Guo et al., 2006), thereby reducing the risk to human and animal health. Finally, plant growth has a beneficial effect on soil fertility and health, thus helping ecosystem restoration.

Nonetheless, as metal pollutants are not removed during phytostabilization processes, long-term monitoring programs are needed to check for metal mobilization, bioavailability and, above all, toxicity and ecological impact. For instance, phytostabilized metal pollutants might become bioavailable with time due to changes in environmental conditions.

The proper selection of plants is a key factor for the successful implementation of phytostabilization in abandoned mine sites. In this respect, plant candidates for metal phytostabilization must, among other features, be well-adapted to high metal concentrations in soil, preferably display a metal excluder phenotype, show tolerance to the stressing soil conditions typical of mining sites, present high rates of growth, have a profuse root system, and be resistant to herbivores and pathogens (Alkorta et al., 2010). When possible, native plants from the specific mining site to be remediated should be used, in order to avoid the introduction of foreign species into the ecosystem (Barrutia et al., 2011) as well as to ensure their optimum adaptation to the site conditions.

Rhizosphere processes and their proper management are crucial for the success of phytostabilization processes. However, the role of rhizosphere processes and mechanisms in the phytoremediation of metals and metalloids have not been thoroughly investigated (Wenzel, 2009).

Chemophytostabilization, also called aided phytostabilization (Alvarenga et al., 2009a,b; Córdova et al., 2011), which is based on the combination of the addition of organic or inorganic amendments with phytostabilization (Knox et al., 2000), is a promising alternative for the remediation of metal-polluted mine soils. Most importantly, chemophytostabilization involves the reutilization of wastes that otherwise would be incinerated, landfilled or applied to agricultural lands, with potential negative environmental consequences. Moreover, the incorporation of organic amendments to the soil, together with the establishment of a vegetation cover might contribute to carbon storage and sequestration as a tool to mitigate global warming (Mench et al., 2009).

Organic amendments, such as farmyard manures, have been used as *in-situ* immobilizing agents to reduce metal bioavailability, through sorption and precipitation processes with organic matter, phosphorous and iron from such amendments (Brown et al., 2003; Singh et al., 2010; Zhou and Haynes, 2010). Organic compounds provide additional binding sites for metal pollutants, while

promoting plant and microorganism development due to the input of organic matter and associated nutrients.

In any case, a successful chemophytostabilization process must lead to the establishment of long-term plant communities, enhance biogeochemical cycles, stabilize microbial communities, maintain soil ecosystem services, and reduce linkages between the polluted soil and humans and animals (Mench et al., 2009).

1.4. The recovery of soil health

The aim of a (chemo)phytostabilization process must not only be to reduce metal mobility and bioavailability, but also to restore soil quality/soil health (Epelde et al., 2009a,b; Hernández-Allica et al., 2006). Soil health can be defined as "the capacity of a soil to perform its ecosystem processes and services, while maintaining ecosystem attributes of ecological relevance" (Garbisu et al., 2011). A set of ecologically-relevant indicators of soil health, which must take into consideration its physical, chemical and biological properties, is then needed for a proper assessment of the effectiveness of metal phytostabilization strategies.

Traditionally, emphasis has been placed on physical (e.g., texture, bulk density, porosity, water holding capacity) and chemical (e.g., pH, organic matter content, macro and microelements) properties as indicators of soil health. However, physicochemical data alone are not sufficient to properly assess the impact of metals on soil health, as they do not take into account the possible effects of metal pollutants on soil organisms or the interactions between metal pollutants, the soil matrix, and biota (Brown et al., 2005; Leitgib et al., 2007). Recently, soil microbial properties have received increased attention as biological indicators of the impact of disturbances on soil health, due to their quick response, high sensitivity, ecological relevance and capacity to provide information that integrates environmental factors (Mijangos et al., 2006, 2010). Microorganisms can provide a direct measure of soil functioning (Bloem et al.,

2006; Ritz et al., 2009) as they have a key role in the delivery of essential soil ecosystem processes and services (Jeffery et al., 2010), and several excellent reviews have been published on the use of microbial properties as indicators of soil health (Arias et al., 2005; Bloem et al., 2006; Ritz et al., 2009; Schloter et al., 2003).

Microbial properties that provide information on the biomass, activity and diversity of soil microbial communities are promising as ecologically-relevant biological indicators of soil health, as well as biomonitoring parameters for the evaluation of soil health recovery during metal phytoremediation (Epelde et al., 2008a,b; 2009a,b; Hernández-Allica et al., 2006). Some microbial properties most commonly used as indicators of soil health are: basal respiration, substrate-induced respiration, potentially mineralizable N, enzyme activities, community-level physiological profiles (Epelde et al., 2008b), microbial biomass C, ATP, nitrification rates, etc. Undoubtedly, the recent innovative breakthroughs in genotypic profiling, ultrahigh-throughput DNA sequencing methods, metagenomics, metatranscriptomics, metaproteomics, metabolomics, and bioinformatics (de Bruijn, 2011a,b) will provide novel and more detailed insights into the functioning of soil microbial communities and their capacity to respond to metal pollution and remediate polluted sites (Desai et al., 2010).

An essential aspect of the use of soil microbial properties as biomonitoring tools to track the effectiveness of phytoremediation is the standardization of the methods used to determine such properties. In any event, soil microbial properties are by definition highly context-dependent and therefore potentially difficult to interpret, which hampers their utilization as indicators of the impact of disturbances (e.g., metal pollution) on soil health. For a better interpretation of microbial properties as indicators of soil health, it has been proposed to group them within categories of higher ecological relevance such as, for instance, soil functions, ecosystem health attributes, or ecosystem services (Garbisu et al., 2011).

1.5. Plant bioassays and biomarkers for phytotoxicity assessment

Plant parameters can also be used as biological tools for phytotoxicity assessment and phytoremediation processes biomonitoring. In fact, plants are primary consumers and are exposed to soil contamination directly and strongly affected by soil composition. Alterations on growth and various physiological and metabolic processes may reflect the presence of toxic substances. Previous studies of our group have described an increased in α-tocopherol levels as a consequence of metal toxicity in plants and stress tolerance (Artetxe et al., 2002; Epelde et al., 2010). In the same way, effects on pigments composition have also been observed: chlorosis symptoms (Artetxe et al., 2002) and deepoxidation index [anteraxanthin + zeaxanthin / (violaxanthin + anteraxanthin + zeaxanthin)] increase (Epelde et al., 2010). Moreover, it has also been described the inhibition by toxic metals of various plant enzymes, such as δ-aminolevulinic acid dehydratase (ALAD), an enzyme of the biosynthetic pathway of porphyrins responsible for chlorophyll synthesis (Cenkci et al., 2010).

Moreover, the development of toxicity tests with living organisms have become an urgent necessity because the priority of some governments concerning soil pollution is to assess the level of risk in order to implement measures to protect ecosystems. Actually, ecotoxicity bioassays aim to provide a realistic prediction of the behavior of contaminants in the environment, identifying contamination hazards. Furthermore, an ecotoxicological approach using biological tests on target organisms at different trophic levels has been recommended for the assessment of environmental hazards as a complement of chemical analyses (Plaza et al., 2005). Cress, barley and lettuce are some examples of the most popular plant species used in bioassays (Boluda et al., 2011).

Although basic research about the phytotoxic effects of metals is widely extended among the scientific community, the development of new consistent and realistic biological indicators of phytotoxicity still remains necessary.

1.6. Oxidative stress, prevention and antioxidant system in plants

Plant biomarkers can be related to the physiological response of plants to reactive oxygen species (ROS) generated as a result of accumulation of toxic metal concentrations in their tissues. Plant species, as other living organisms, have developed mechanisms to reduce ROS production, but, when the capacity to prevent or face this damage is insufficient for cell protection, oxidative stress is triggered. Non-redox metals like Cd, Pb and Zn can generate oxidative stress in plants by altering the cellular redox state indirectly through the interference with components of the respiratory chain, the photosynthethic apparatus or the antioxidant system (Bi et al., 2009; Jozefczak et al., 2012). Two systems operate sequentially and prevent or protect from oxidative damage: (i) carotenoids, including xanthophylls and β-carotene, and (ii) antioxidant systems (Fig. 1.1). First of all, in order to prevent ROS and free radicals generation, the VAZ pigments (violaxanthin, antheraxanthin and zeaxanthin) dissipate and quench excess light energy received by the plant (Fig. 1.2). Secondly, antioxidant systems are responsible for free radicals and ROS quenching through non-enzymatic molecules and enzymatic reactions (Fig. 1.3) in order to prevent membrane lipids, proteins and nucleic acids damage.

1.7. Lead effect on porphyrins biosynthetic pathway

Photosynthethic organisms synthetize clorophylls, heme groups, syroheme and bilins through the porphyrins biosynthetic pathway, also known as tetrapyrroles pathway (Fig. 1.4). Animals also use this pathway for heme synthesis. The substrate δ-aminolevulinic acid (δ-ALA) is the precursor of all macrocyclic and linear tetrapyrroles. Two pathways exist for its generation: the C_5 (plants, algae, cianobacteria and some bacteria) and the Shemin pathway (animals and some bacteria) (Fig. 1.4) (Vavilin y Vermaas, 2002).

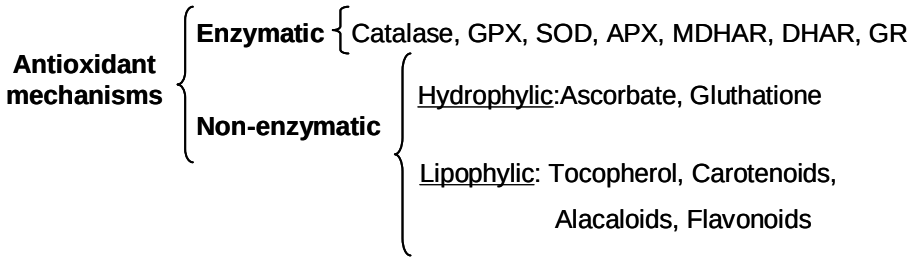

Fig. 1.1. Antioxidant mechanisms. GPX: glutathione peroxidase; SOD: superoxide dismutase; APX: ascorbate peroxidase: MDHAR: monodehydroascorbate oxidase; DHAR: dehydroascorbate oxidase; GR: glutathione reductase.

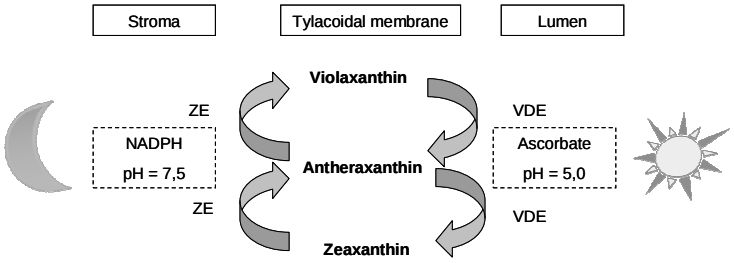

Fig. 1.2. Xanthophylls cycle. VDE: violaxanthin deepoxidase, ZE: zeaxanthin epoxidase, Ascorbate and NADPH are cofactors. Wigh light, V is transformed to A and Z, which is implicated in thermal dissipation of light energy (non photochemical quenching induction). In the dark, A and Z are epoxidized to V which allow a higher quantum yield.

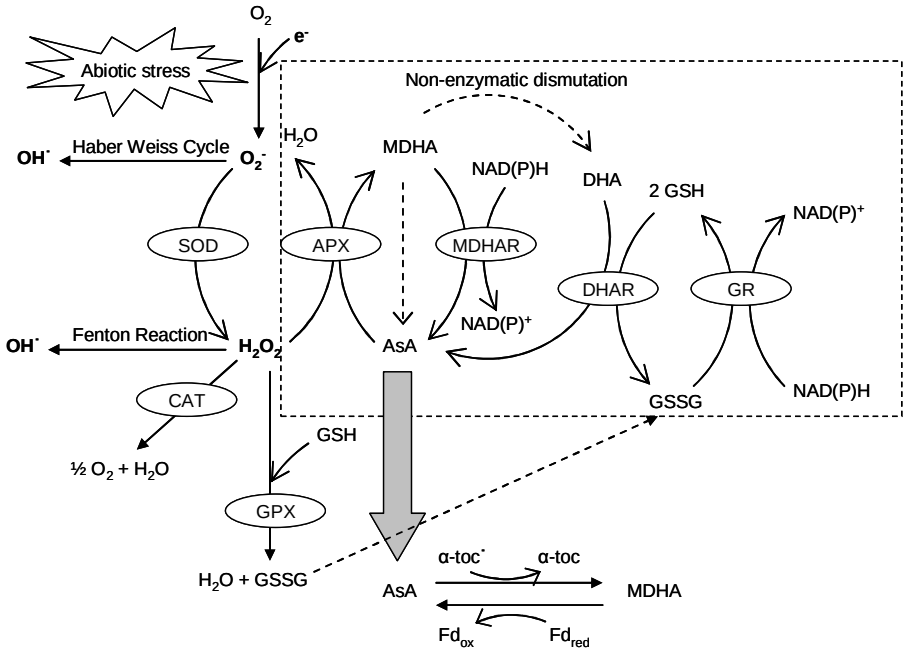

Fig.1.3. Antioxidant mechanisms in plants for H_2O_2 elimination in chloroplasts. The ascorbate-glutathione cycle, also known as Halliwell-Asada cycle, is defined by a discontinuous line. APX: ascorbate peroxidase; CAT: catalase; DHA: dehydroascorbate; DHAR: DHA reductase; Fd_{ox}: oxydized ferredoxin; Fd_{red}: reduced ferredoxine; GPX: glutathione peroxidase; GR: glutathione reductase; GSH: reduced glutathione; GSSG: oxidized glutathione; MDHA: monodehydroascorbate; MDHAR: MDHA reductase; $NAD(P)^+$: oxidized nicotinamide adenine dinucleotide phosphate; NAD(P)H: reduced nicotinamide adenine dinucleotide phosphate; SOD: superoxide dismutase; α-toc: α-tocopherol; α-$toc^·$: α-tocopheroxyl radical.

In animals, it has been described that Pb can inhibit δ-aminolevulinic acid dehydratase (ALAD) resulting in the accumulation of δ-ALA which is converted to 4,5-dioxovaleric acid (DOVA), responsible for ROS production (Bechara, 1996). Both DOVA and ROS generate oxidative stress (Bechara et al., 2007). In plants, it has also been described that non-essential metals such as Cd, Pb or Hg can inhibit ALAD, hampering chlorophyll synthesis and leading to δ-ALA accumulation

(Calgaroto et al., 2010; Cenkci et al., 2010; Gonçalves et al., 2009; Lenti et al., 2002; Prasad y Prasad, 1990; Stobart et al., 1985). However, it remains unknown which are the consequences of δ-ALA accumulation in plants.

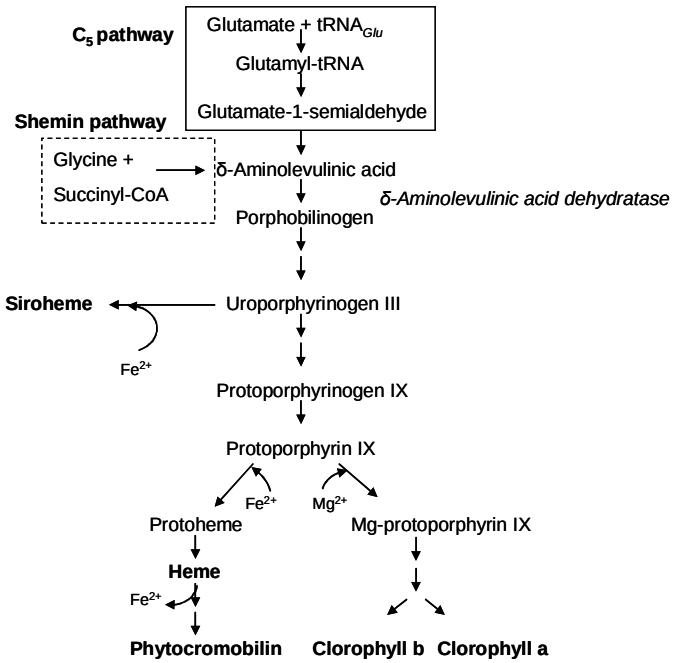

Fig. 1.4. Porphyrins biosynthetic pathway in photosynthetic and heterotrophic organisms. Only compounds synthesized by plants are represented (adapted from Moulin and Smith, 2005 and Vavilin and Vermaas, 2002).

1.8. Soil contamination in the Autonomous Community of the Basque Country

According to the Spanish Royal Decree RD 2994/1982 (repealed by RD 975/2009), mine owners are responsible for land restoration once mining activities finish. But, exploitations whose activities ceased before this norm was published, remain abandoned and unrestored ("orphaned mines"). This is the case of some mines located in the Biscay province of the Autonomous Community of the Basque Country where minerals of iron, zinc, lead, copper and

barium were exploited during the 20th century (IGME, 1980). According to the "Inventario de Emplazamientos con Actividades Potencialmente Contaminantes del Suelo de la Comunidad Autónoma del País Vasco" (2008), there are between 1,279 and 3,120 ha altered or contaminated sites in the Basque Country (local contamination) and metals are some of the most abundant contaminants in soils and underground water (34%). However, few data is available for the assessment of diffuse soil contamination, where the massive use of fertilizers and pesticides in agricultural soils is included.

In order to face local contamination issue, IHOBE, S.A., a public corporation for environmental management in the Basque Country, has published some Indicative Values of Evaluation (VIE) for ecosystem protection (Table 1.1): (i) VIE-A is the lower reference limit at which soil is considered as non-contaminated and no risk for health exists; (ii) VIE-B is the upper limit concentration under which the risk is acceptable, and (iii) VIE-C is the upper limit concentration above which the risk is unacceptable. In the same way, this public corporation published the "Plan de Suelos Contaminados de la Comunidad Autónoma del País Vasco (2007-2012)" (IHOBE, 2008), the first autonomic program for soil contamination prevention and soil restoration.

Table 1.1. Indicative Values of Evaluation of cadmium, lead and zinc for ecosystem protection in the Basque Country (IHOBE, 1994, 1998).

	Cadmium	**Lead**	**Zinc**
VIE-A	0,17 + 0,013 L	16 + 0,7 L + 2,1 H	50 + 2 L
VIE-B	0,8	44	106
VIE-C	18	330	840

L: clay content (%); H: organic matter content (%)
Units: mg kg^{-1}

In spite of European, Spanish national and autonomic regulations, soil contamination and thus, degradation, continue increasing, being the development of cost-effective and environmentally friendly techniques for soil remediation urgent. In this regard, the growing public and commercial interest in

phytoremediation technologies and the success of some trials and large-scale field studies have allowed a higher development of phytoremediation processes, although more investigation on metalliferous plants, soil-roots-microbial interactions (Vangronsveld et al., 2009) and soil and plant bioindicators of soil health and plant performance for monitoring of phytoremediation processes is necessary.

2. Objectives

Volverán las tupidas madreselvas
de tu jardín las tapias a escalar,
y otra vez a la tarde, aun más hermosas,
sus flores se abrirán;
pero aquéllas, cuajadas de rocío,
cuyas gotas mirábamos temblar
y caer, como lágrimas del día...
ésas... ¡no volverán!

(Gustavo Adolfo Bécquer)

2. Hypothesis and Objectives

2.1. Hypothesis

Phytostabilization is a promising technology for the remediation of contaminated soils with high levels of metals, but requires the implementation of efficient plant species, amendments and relevant indicators of the effectiveness of the process. We consider that excluder species native from mining soils and organic livestock amendments could improve physiochemical and biological soil properties, not only in terms of reducing metal mobility, but also in terms of recovery of soil health. Plant and microbial bioindicators can be complementary tools to assess effectiveness of phytostabilization processes.

2.1. General objective

The general objective of this thesis is to assess the phytostabilization process of a Pb/Zn mine soil with organic amendments and the native excluder species, *Festuca rubra,* using physicochemical and biological indicators. The identification of new bioindicators of metal phytotoxicity is also considered.

2.2. Specific objectives

- Study of the early soil-amendment interactions responsible for the stabilization-induced changes in soil physicochemical and biological properties, and the impact of microbial populations present in organic amendment on soil communities of mine soil.

- Assessment of the efficiency of organic amendments combined with a native metallicolous population of *Festuca rubra* L., in order to select the most effective amendment for further on-site aided phytostabilization of a Pb/Zn contaminated mine soil, in terms of reduction of metal bioavailability and phytotoxicity, and improvement of soil health.

- Carry out a field study on a Pb/Zn mine area in order to:

    - Assess the effectiveness of different organic amendments for the on-site phytostabilization of vegetated and non-vegetated sites.

    - Assess the success of direct revegetation of bare soils with native plants.

    - Assess the suitability of several chemical and biological indicators (plant and microbial parameters) to monitor aided phytostabilization processes and soil health recovery.

- Relevance of plant based bioassays for aided phytostabilization monitoring programs. Exploration of new plant biomarkers: porphyrin inhibition caused by Pb and Cd, and the role of δ-aminolevulinic acid on phytotoxicity and antioxidant response of plants to heavy metals.

3. Materials and methods

Como no sabían que era imposible, lo hicieron.

3. Materiales y Métodos

3.1. Localización y características del entorno minero

La zona de estudio se encuentra en Carranza, al oeste de la provincia de Bizkaia, en la Comunidad Autónoma del País Vasco, al norte de España (43º13' N; 3º26' O) (Fig. 3.1).

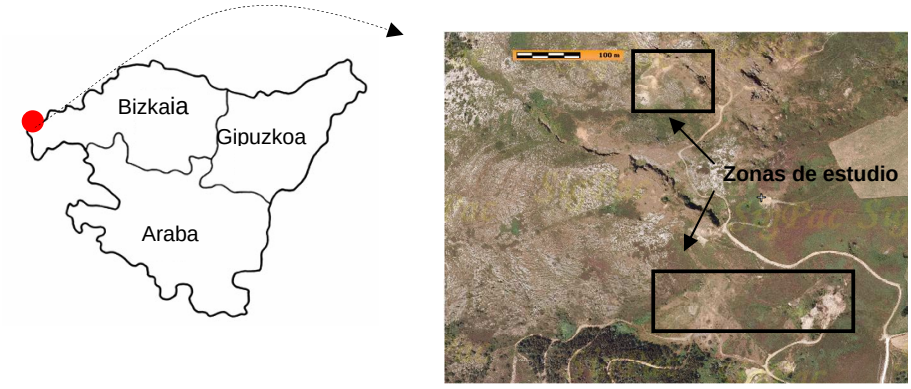

Fig. 3.1. Localización y ortofoto del entorno minero estudiado.

Se trata de una mina subterránea abandonada, conocida como "Coto Txomin", que se encuentra en la vertiente meridional del monte Moro y cuya actividad cesó a finales de de la década de 1970. La zona posee un clima templado húmedo, también denominado clima atlántico, caracterizado por presentar temperaturas moderadas durante todo el año y ser muy lluvioso (Euskalmet, 2010). La zona de estudio se encuentra a una altitud de entre 560 y 640 m, cuya vegetación potencial estaría representada por las series predominantes del piso mesotemplado (*Polysticho setiferi-Fraxino excelsioris-S*., *Hyperico pulchri-Querco roboris-S*., *Melampyro pratensis-Querco pyrenaicae-S*., *Lauro nobilis-Querco ilicis-S*. e *Hyperico androsaemi-Alno-S*.) y que actualmente, debido a la acción humana, han sido sustituidas por prados de siega, brezal-argomales, saucedas con abedules, zarzales, etc., y cultivos madereros de *Pinus*

radiata y *Eucalyptus globulus* (Biurrun y cols., 2011) en las zonas adyacentes al entorno minero de estudio. Durante el ensayo de campo (del 17 de marzo al 21 de septiembre de 2010), las temperaturas mínima y máxima fueron de 1,1 ºC en mayo y 33,5 ºC en agosto, respectivamente, con una temperatura media de 11,2 ºC en primavera y 17,1 ºC en verano y una precipitación total de 193 l m^{-2} en primavera y 103 l m^{-2} en verano (datos procedentes de la estación de aforo del embalse de La Cerroja, en Carranza, situada a 1,4 km de la zona de estudio y a 678 m de altitud). En la zona se encuentran depósitos metalíferos de Pb y Zn de tipo Valle del Mississippi (Velasco y cols., 1994) y el depósito hipogénico consiste en esfalerita y galena (los dos minerales principalmente extraídos), pirita, dolomita, calcita y cuarzo (Grandia y cols., 2003). En el entorno minero se pueden encontrar pilas de rocas, superficies quemadas, diques de sedimentación, hundimientos y zonas degradadas sin vegetación, entre otros. Se trata de una zona revegetada de forma natural una vez finalizados los trabajos mineros. Una descripción más detallada de la zona de estudio ha sido publicada por Barrutia y cols. (2011).

3.2. Caracterización del suelo minero y de las enmiendas orgánicas

Las enmiendas orgánicas empleadas en los estudios en microcosmos (Tabla 3.1) y en el estudio de campo (Tabla 3.2) procedían de diversas empresas: (i) los purines bovinos de la empresa “Ganado Miren Ardeo” de Laukiniz (Bizkaia); (ii) la gallinaza semiseca de la granja “Avícola Arbaraitz” de Egino (Asparrena, Bizkaia); (iii) los lodos de papelera de la empresa Papresa (Rentería – Gipuzkoa) mezclados con gallinaza de la granja “Avícola Gorrotxategi” (Idiazabal – Gipuzkoa), y (iv) el estiércol de oveja (raza Latxa, variedad Cara negra) del rebaño experimental de Neiker-Tecnalia-Arkaute (Álava).

El suelo empleado en los capítulos 4, 5 y 6 (Tabla 3.3) procedía del entorno minero descrito en el apartado 3.1.

Los parámetros químicos de enmiendas y suelo fueron determinados según los métodos oficiales de análisis publicados por la Secretaría General Técnica del Ministerio de Agricultura, Pesca y Alimentación (MAPA, 1994) por el laboratorio químico de análisis agrícola MOPRILAB, S.L. (Abarán, Murcia).

3.3. Determinación de metales

3.3.1. Digestión de metales totales en suelo

Los suelos se tamizaron a 2 mm con la ayuda de un cedazo y, una vez eliminadas las raíces, se secaron en estufa y conservaron a temperatura ambiente hasta su análisis.

El contenido total de Cd, Pb y Zn del suelo se determinó mediante digestión con *aqua regia* según el método descrito por McGrath y Cunliffe (1985). Se añadieron 4 ml de HCl (32%) y 1 ml de HNO_3 (65%) de alta calidad (pureza ≥ 98,5%) a 250 mg de muestra de suelo seco en un tubo de vidrio. Tras una predigestión mínima de 4 horas, se digirió la muestra en un bloque metálico calefactor (Bloc Digest m 40, Selecta) controlado por un programador de procesos tiempo/temperatura (RAT-2, Selecta) según la siguiente rampa de temperaturas: 3 h a 60 ºC, 1 h a 105 ºC, 2 h a 125 ºC y dejar enfriar.

Tabla 3.1. Caracterización completa de las enmiendas de los ensayos en microcosmos recogidas en abril de 2008. Valores expresados por peso seco.

Parámetro	Gallinaza	Lodos de papelera con gallinaza (2:1)	Estiércol ovino	Purín bovino
pH (1:10)	7,6	8,2	9,1	6,1
CE (mS cm^{-1}) (1:10)	6,15	1,85	15,54	16,91
% Humedad	21,24	35,54	71,88	91,15
% MO total	73,10	33,10	62,71	79,74
% MO oxidación	54,37	28,66	38,87	71,38
% Cenizas	26,90	66,90	37,19	20,26
% Ácidos húmicos	3,67	1,05	4,41	2,99
% Ácidos fúlvicos	6,43	1,42	2,84	8,19
Relación C/N	5,94	28,45	8,32	17,77
% $CaCO_3$ total	15	40	14	5
% Nitrógeno total (N)	5,31	0,58	2,71	2,33
% Potasio total (K_2O)	3,33	0,65	7,14	3,51
% Calcio (CaO)	9,08	27,76	7,53	3,52
% Magnesio (MgO)	0,91	0,60	1,26	1,24
% Fósforo total (P_2O_5)	5,62	0,90	3,47	1,29
% Azufre (SO_3)	0,63	0,46	1,03	0,47
% Hierro (Fe)	0,10	0,12	0,17	0,16
% Sodio (Na)	0,27	0,12	0,83	1,37
B (mg kg^{-1})	50,1	19,1	61,6	48,1
Mn (mg kg^{-1})	227,2	125,9	259,4	273,2
Zn (mg kg^{-1})	487,2	325,0	482,6	524,4
Cu (mg kg^{-1})	22,8	154,5	21,4	28,3
Ni (mg kg^{-1})	4,38	4,48	7,00	3,73
Se (mg kg^{-1})	2,51	1,13	1,78	5,00
As (mg kg^{-1})	0,52	3,17	2,30	0,78
Cd (mg kg^{-1})	0,21	0,18	0,22	0,12
Cr (mg kg^{-1})	3,06	18,28	12,88	2,55
Pb (mg kg^{-1})	0,65	16,40	3,39	2,61

CE: conductividad eléctrica
MO: materia orgánica

Tabla 3.2. Caracterización completa de las enmiendas del ensayo en campo recogidas en enero de 2010. Valores expresados por peso seco.

Parámetro	Gallinaza	Lodos de papelera con gallinaza (2:1)	Purín bovino
pH (1:10)	7,9	7,7	5,9
CE (mS cm^{-1}) (1:10)	6,52	3,66	11,6
% Humedad	26,6	66,3	90,7
% MO total	73,5	51,0	86,0
% MO oxidación	56,8	48,0	78,0
% Cenizas	26,5	49,0	14,0
% Ácidos húmicos	3,59	1,83	2,02
% Ácidos fúlvicos	7,55	2,83	7,70
Relación C/N	7,60	29,2	25,0
% $CaCO_3$ total	16	30	3
% Nitrógeno total (N)	4,33	0,95	1,81
% Potasio total (K_2O)	3,34	1,41	2,54
% Calcio (CaO)	9,29	18,2	2,43
% Magnesio (MgO)	0,85	1,19	0,74
% Fósforo total (P_2O_5)	4,01	2,80	1,14
% Azufre (SO_3)	0,48	0,65	0,40
% Hierro (Fe)	0,07	0,11	0,09
% Sodio (Na)	0,28	0,21	0,71
B (ppm)	48,2	29,6	35,7
Mn (ppm)	238	242	173
Zn (ppm)	266	353	253
Cu (ppm)	32,0	64,8	17,7
Ni (ppm)	3,52	9,59	4,38
Se (ppm)	2,47	1,29	2,51
As (ppm)	0,76	1,84	0,52
Cd (ppm)	1,21	0,42	0,21
Cr (ppm)	3,22	2,23	3,06
Pb (ppm)	9,45	13,3	0,65

CE: conductividad eléctrica

MO: materia orgánica

Tabla 3.3. Caracterización fisicoquímica del suelo minero del ensayo en microcosmos (capítulos 4, 5 y 6). Valores expresados por peso seco de suelo.

Parámetro	Suelo	Parámetro	Suelo
pH (1:5)*	5,7	**Na asimilable (ppm)**	59,8
CE (µS cm^{-1}) (1:5)*	19,1	**B asimilable (mg kg^{-1})**	0,39
CIC (meq 100 g^{-1})	1,52	**B total (mg kg^{-1})**	16,8
% MO total	5,67	**Mn asimilable (mg kg^{-1})**	0,2
% MO oxidable	4,36	**Mn total (mg kg^{-1})**	114
% Ácidos húmicos	1,71	**Zn asimilable (mg kg^{-1})**	841
% Ácidos fúlvicos	0,31	**Zn bio (mg kg^{-1})***	531
Relación C/N	14,4	**Zn total (mg kg^{-1})***	17.576
% Carbono orgánico total	3,29	**Cu asimilable (mg kg^{-1})**	0,1
% Carbonatos totales	4	**Cu total (mg kg^{-1})**	12,8
% N total	0,229	**Ni total (mg kg^{-1})**	8,84
K asimilable (mg kg^{-1})	58,7	**Se total (mg kg^{-1})**	1,52
Ca asimilable (mg kg^{-1}	281	**As total (mg kg^{-1})**	9,76
Mg asimilable (mg kg^{-1})	62	**Cd bio (mg kg^{-1})***	0,44
P asimilable (mg kg^{-1})	29,7	**Cd total (mg kg^{-1})***	4,99
P total (P_2O_5) (mg kg^{-1})	526	**Cr total (mg kg^{-1})**	14,5
Sulfato (SO_4) (mg kg^{-1})	28,8	**Pb bio (mg kg^{-1})***	125
Fe asimilable (mg kg^{-1})	910	**Pb total (mg kg^{-1})***	34.692
Fe total (mg kg^{-1})	9.109		

CIC: capacidad de intercambio catiónico
MO: materia orgánica
bio: biodisponible
* Parámetros determinados en el laboratorio de Fisiología Vegetal de la UPV/EHU

A continuación, se añadieron 5 ml de HNO_3 al 20% al tubo, se agitó y se recalentó a 80 ºC durante 30 min. Posteriormente, se añadieron 14 ml de agua MilliQ, se agitó la muestra y se recalentó a 80 ºC durante 30 min. Una vez frío, se enrasó el tubo a 20 ml con agua MilliQ y se agitó. La muestra se filtró con un filtro Whatman nº 40.

3.3.2. Digestión de metales totales en planta

Se empleo el método descrito por Zhao y cols. (1994) para la determinación de Cd, Pb y Zn en plantas. Se añadieron 5 ml de una solución ácida compuesta por HNO_3 y $HClO_4$ (85:15) de alta calidad (pureza ≥ 98,5 %) a 250 mg de hojas o raíces finamente cortadas y secas en tubos de vidrio. Tras una predigestión mínima de 4 horas, la digestión de las muestras se llevó a cabo en un bloque metálico calefactor (Bloc Digest m 40, Selecta) controlado por un programador de procesos tiempo/temperatura (RAT-2, Selecta) según la siguiente rampa de temperaturas: 3 h a 60 ºC, 1 h a 100 ºC, 1 h a 120 ºC, 2,5 h a 195 ºC y dejar enfriar. Una vez finalizada la digestión, se añadieron 5 ml de HNO_3 al 20% a los tubos, se agitaron y se recalentaron a 80 ºC durante 30 minutos. A continuación, se añadieron 14 ml de agua MilliQ a las muestras, se agitaron y se recalentaron de nuevo a 80 ºC durante 30 min. Una vez fría la mezcla, se enrasaron los tubos a 20 ml con agua MilliQ y se agitaron. Por último, las muestras se filtraron con filtros Whatman nº 40.

3.3.3. Extracción de metales biodisponibles en suelo

La concentración de metales biodisponibles en el suelo se determinó siguiendo el método descrito por Houba y cols. (2000) que emplea $CaCl_2$ como extractante, el cual posee una fuerza iónica similar a la concentración salina media de muchos suelos y es capaz de extraer los cationes absorbidos por éste.

La concentración electrolítica de la muestra se mantiene prácticamente constante, la concentración del metal refleja las diferencias en la fuerza de unión y/o en la solubilidad de este metal entre varios suelos, y el elemento determinado refleja su disponibilidad según el pH del suelo ya que el extractante no está tamponado (Jones, 2001). Siguiendo este procedimiento, se agitó durante 2 horas una suspensión de suelo seco tamizado a 2 mm y $CaCl_2$ 0,01 M en una proporción 1:10 (p/v). A continuación, se filtró la mezcla a través de filtros Whatman nº 42 y se añadió HNO_3 (concentración final inferior al 5%) para evitar la precipitación de los metales.

3.3.4. Determinación de metales

Las muestras de suelo o planta digeridas y los extractos de suelo se conservaron hasta un máximo de 7 días en oscuridad a 4 ºC hasta su análisis por ICP-AES (Espectrometría de Emisión Atómica por Plasma Inductivamente Acoplado) (Horiba Jobin Yvon modelo Activa) por el Servicio Central de Análisis de los SGIker de la UPV/EHU (Unidad de Bizkaia).

3.4. Determinación de parámetros microbiológicos del suelo

Las muestras de suelo frescas, tamizadas a 2 mm, se conservaron en oscuridad a 4 ºC durante 1 máximo de un mes antes de su análisis.

3.4.1. Actividades enzimáticas

La actividad potencial máxima de **β-glucosidasa** (EC 3.2.1.21), **arilsulfatasa** (EC 3.1.6.1) y **fosfatasa ácida** (EC 3.1.3.2) se determinó según Dick y cols. (1996) y Taylor y cols. (2002). Se añadieron 1,6 ml de tampón

(tampón universal modificado MUB 20 mM, pH 6; tampón acetato 0,5 M, pH 5,8 y tampón MUB 20 mM, pH 6,5, respectivamente) y 0,4 ml de sustrato (1,5% p/v de 4-nitrofenil β-D-glucopiranósido, 1,29% p/v de 4-nitrofenil sulfato de potasio y 1,85% p/v de 4-nitrofenil fosfato disódico hexahidratado, respectivamente) a 1 g de suelo fresco tamizado a 2 mm. La mezcla se incubó en un tubo a 37 ºC durante 45 min y la reacción se detuvo con 0,4 ml de $CaCl_2$ 0,5 M y 1,6 ml de NaOH 0,5 M (se empleó tampón TRIS 0,1 M, pH 12 en vez de NaOH para la actividad β-glucosidasa). Tras la centrifugación de la muestra (3.500 rpm, 5 min), se determinó la absorbancia del sobrenadante a 410 nm con un espectrofotómetro Multiskan Spectrum (Thermo Electron Corporation). Una concentración 0,1 M de tampón MUB contenía tampón TRIS 0,1 M, ácido maleico 86,5 mM, ácido cítrico 66,6 mM, ácido bórico 101,9 mM y NaOH 488 mM, mientras que el tampón acetato contenía acetato sódico trihidratado 0,5 M y un 0,17% de ácido acético glacial.

La actividad **ureasa** (EC 3.5.1.5), relacionada con el ciclo del nitrógeno, se determinó según el método descrito por Kandeler y Gerber (1988). Se añadieron 1,75 ml de tampón borato 0,1 M (pH 10) y 0,25 ml de una solución de urea 820 mM a 1 g de suelo fresco tamizado a 2 mm. La mezcla fue incubada a 37 ºC durante 1 h y la reacción se detuvó con 6 ml de KCl 2 M acidificado. Tras la centrifugación de la muestra (3.500 rpm, 5 min), se mezclaron 0,25 ml de sobrenadante con 3,75 ml de agua desionizada y 2 ml de una mezcla de salicilato sódico 0,35 M, nitroprusiato sódico 1,34 mM y NaOH 0,1 M. Por último, se añadieron 0,8 ml de dicloroisocianurato sódico 3,9 mM. Se dejó reposar la muestra durante 30 min y se midió la absorbancia a 670 nm. El tampón borato se preparó con tetraborato sódico decahidratado y sosa 3 M para el ajuste del pH.

La actividad hidrolítica de la **fluoresceína diacetato** (FDA), estimada según García y cols. (1993), Taylor y cols. (2002) y Shaw y Burns (2006), indica la presencia de bacterias y hongos descomponedores en suelos mediante la determinación de grupos enzimáticos (esterasas, proteasas y lipasas) involucrados en la descomposición de tejidos y que son capaces de hidrolizar la

FDA. La actividad hidrolítica de la FDA se determinó mezclando 1 g de suelo fresco tamizado a 2 mm con 4 ml de tampón Tris 0,1 M (pH 7,6) y 50 µl de una solución de fluoresceína diacetato en acetona (0,2% p/v). La mezcla se incubó durante 12 min a 25 ºC y la reacción se detuvo con 4 ml de acetona. Tras la centrifugación de la muestra (14.000 rpm, 5 min), se determinó su absorbancia a 490 nm.

3.4.2. Respiración basal y respiración inducida por sustrato

Para llevar a cabo la determinación de la **respiración basal** de las muestras (Norma ISO 16072, 2002), se introdujo el equivalente a 30 g de peso seco de suelo en un tarro de vidrio con cierre hermético junto a un vial con 10 ml de NaOH de 0,2 M a 0,8 M. Se incubaron las muestras en oscuridad a 30 ºC durante 72 horas en una cámara de incubación (Sanyo Incubator). Transcurridas 72 horas, se añadieron 4 ml de $BaCl_2$ 0,5 M y unas gotas de fenolftaleína 0,1% (en etanol) a los viales con NaOH para valorar la cantidad restante con HCl 0,1 M. A partir de estos datos, se calculó la cantidad de carbono generada en forma de CO_2 procedente de la respiración de los microorganismos del suelo.

Para la determinación de la **respiración inducida por sustrato** (Norma ISO 17155, 2002) se pulverizó una disolución de glucosa (10.000 ppm de carbono) sobre las mismas muestras de suelo empleadas para la determinación de la respiración basal y se introdujo un nuevo vial con NaOH en los tarros. Se incubaron las muestras de suelo durante 6 horas en oscuridad a 30 ºC y se valoró de nuevo el NaOH restante. Según Lin y Brookes (1999), un tiempo de incubación corto permite registrar la respiración de la población microbiana inicial sin favorecer su multiplicación.

3.4.3. Perfiles fisiológicos de comunidades microbianas (CLPPs)

En nuestros estudios utilizamos el Biolog EcoPlate™ que contiene 31 fuentes de carbono que, en presencia de un indicador redox, tetrazolio (incoloro), se reduce a formazán (color púrpura) cuando el sustrato es consumido.

Todo el material y el agua empleados fueron previamente esterilizados en autoclave a 121 ºC durante 15 minutos. Se añadieron 9 ml de agua MilliQ al equivalente a 1 g de peso seco de cada una de las muestras en tubos de vidrio. Se agitaron los tubos durante 1 hora a 125 rpm en un agitador de vaivén y se dejaron reposar durante 5 minutos. A continuación, se añadieron 19,8 ml de agua MilliQ a 200 µl de cada sobrenadante sobre una placa Petri de vidrio para diluir las muestras. Tras agitar, se inocularon los pocillos del Biolog EcoPlate™ por duplicado con 150 µl de sobrenadante diluido. Las placas se incubaron a 30 ºC en oscuridad en una cámara Sanyo Incubator. El desarrollo de color en los pocillos se midió espectrofotométricamente a 595 nm al inicio y cada 12 horas aproximadamente durante 4 días para determinar el momento en el cual la tasa de crecimiento era máxima. Tras analizar la curva de crecimiento, se eligió la hora 40 como tiempo de comparación entre muestras. Se utilizaron un lector de microplacas (Anthos Zenith 3100) y el programa Standard Detection Software (Anthos Labtec Instruments GMBH, 2004) para la determinación de las absorbancias. A partir de éstas, se determinaron la tasa y extensión del desarrollo de color (AWCD=$(\Sigma x_i - x_0)/n$ donde x_i es la absorbancia media de cada pocillo, x_0 la absorbancia media del pocillo control que contiene agua y n el número de pocillos con fuentes de carbono, en este caso, 31), el número de sustratos utilizados (riqueza de especies), el índice de biodiversidad de Shannon (H'=-Σpilog2pi donde p_i es la proporción entre la absorbancia de cada pocillo y la suma de todas las absorbancias) y la huella metabólica de las comunidades microbianas a partir del consumo de cada uno de los sustratos. Para el cálculo del índice de Shannon y la huella metabólica, solo las absorbancias superiores a 0,25 (ensayos en microcosmos) o 0,1 (ensayo en campo) se consideraron

positivas. Estos parámetros permiten conocer el número de fenotipos presentes en la comunidad microbiana edáfica y la diversidad funcional de ésta.

3.5. Bioensayos de toxicidad del suelo con *Lactuca sativa* L.

Se colocaron 10 g de suelo seco tamizado a 2 mm en una placa Petri (Ø 8,5 cm) cuya tapa había sido previamente agujereada para evitar la condensación de agua en su interior. Se colocó un papel de filtro sobre la muestra de suelo y se humedeció con agua desionizada. Paralelamente, se germinaron 50 mg de semillas de *Lactuca sativa* variedad Reina de Mayo de origen ecológico (marca comercial Clemente Viven) en una placa Petri (Ø 8,5 cm) con papel de filtro humedecido con 3 ml de agua desionizada. Las placas se incubaron durante dos días a 25 ºC (cap. 4 y 5) o 25/18 ºC día/noche (cap. 6) y humedad relativa 50% (cap. 4 y 5) u 80% (cap. 6) en una cámara de incubación (Sanyo Versatile Environmental Test Chamber). Las semillas se germinaron en oscuridad y los suelos se expusieron a una intensidad lumínica de 100 µmol m^{-2} s^{-1} y un fotoperiodo de 14 horas. Transcurridas las 48 horas, se transplantaron 25 plántulas con una radícula de 5 mm de longitud a la placa con suelo y, en caso necesario, se añadió agua desionizada hasta alcanzar aproximadamente un 85% de la capacidad del campo del suelo, asegurando así un contenido hídrico adecuado durante el ensayo. Las plántulas se incubaron en las condiciones anteriormente descritas, con un fotoperiodo de 14 horas, durante 72 horas. Al final del ensayo (Fig. 3.2), se determinó la longitud radicular de las plántulas. Se utilizó una placa con semillas sin suelo como control positivo para comprobar si el crecimiento de la radícula en las condiciones descritas se mantenía en los sucesivos ensayos.

Fig. 3.2. Plántulas de *L. sativa* crecidas sobre un suelo minero contaminado tratado con una enmienda orgánica al final del bioensayo.

3.6. Material vegetal y condiciones de cultivo

Los especímenes de *Festuca rubra* L., gramínea (familia *Poaceae*) conocida como festuca roja, empleados en los ensayos de los capítulos 4, 5 y 6 procedían del entorno minero de la mina "Coto Txomin". Se recogieron cepellones de aproximadamente 15 x 15 x 15 cm del horizonte superficial del suelo donde *F. rubra* predominaba. En el laboratorio, se separaron los clones individuales de *F. rubra*, se limpiaron con agua de grifo, se aclararon con agua desionizada y se sumergieron las raíces durante 30 min en una solución de $CaCl_2$ 0,01 M para eliminar iones metálicos intercambiables. Las plantas se mantuvieron en invernadero (16-26 ºC) en bandejas alveolares con sustrato a base de turba negra durante un mínimo de dos meses antes del ensayo (Fig. 3.3). Las plantas se regaron tres veces por semana.

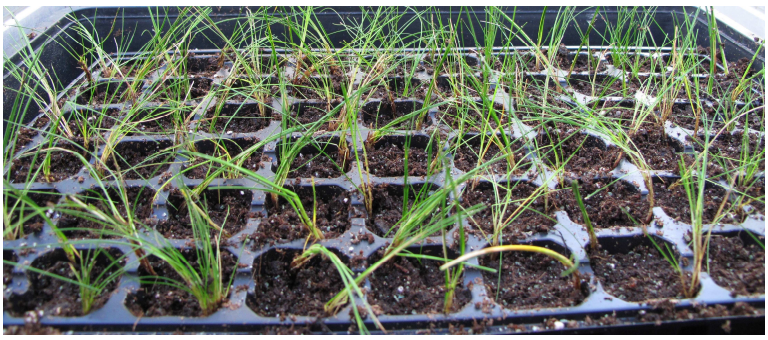

Fig. 3.3. Cultivo de *Festuca rubra* en bandejas alveolares.

Las colonias de *Lemna minor* L. (Fig. 3.4), planta comúnmente conocida como lenteja de agua (familia *Lemnaceae*), empleadas en los ensayos del capítulo 5 procedían de un cultivo mantenido durante los últimos años en el laboratorio de Fisiología Vegetal de la UPV/EHU (Artetxe y cols., 2006). Se cultivaron en el fitotrón del Servicio Fitotrón e Invernaderos de los SGIker de la UPV/EHU a 25/18 ºC y 60/70% de humedad relativa (día/noche), con un fotoperiodo de 14 horas y una PPFD (densidad fotosintética del flujo de fotones) de 300 µmol m^{-2} s^{-1}. Como medio de cultivo se empleó una solución Hoagland modificada (Cross, 2002). Dos veces por semana se renovaba el medio y se retiraban las algas que hubiesen podido aparecer.

Para ajustar la concentración de Pb y Cd libre presente en los medios de ensayo se utilizó el programa Geochem-EZ (www.plantmineralnutrition.net).

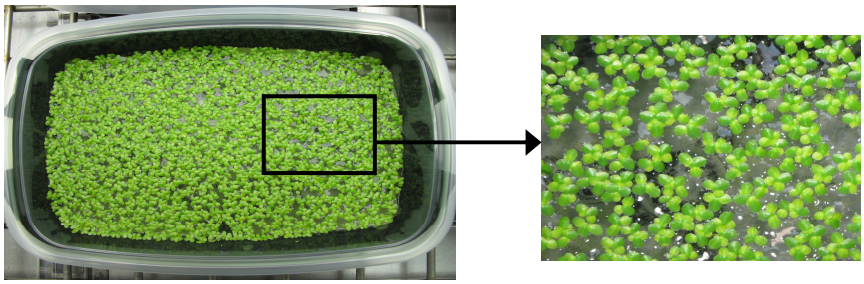

Fig. 3.4. Cultivo de *Lemna minor* y detalle de un conjunto de colonias.

3.7. Determinación de parámetros vegetales

3.7.1. Tasa de crecimiento relativo

La tasa de crecimiento relativo (RGR) de las colonias de *Lemna minor* se calculó de acuerdo a la fórmula [1], donde P_1 y P_2 son los pesos frescos en los tiempos t_1 y t_2, respectivamente.

$$RGR = (\ln P_2 - \ln P_1)/(t_2 - t_1) \quad [1]$$

3.7.2. Enzimas antioxidantes

Se determinaron las actividades antioxidantes superóxido dismutasa (SOD), ascorbato peroxidasa (APX), monodehidroascorbato reductasa (MDHR) y glutatión reductasa (GR) en plantas de *Lemna minor*.

Para la extracción de las enzimas se adaptó el método de Polle y cols. (1993). Se emplearon colonias de *L. minor* previamente congeladas a -80 ºC durante un máximo de 6 semanas. Se homogeneizaron 100 mg de PF con 5 ml de tampón fosfato 100 mM (pH 7,8) con Tritón X-100 al 2% (p/v) y 200 mg de polivinilpolipirrolidona en un mortero previamente enfriado con nitrógeno líquido. Se centrifugó el homogenado a 16.100 g y 4 ºC durante 30 min. Para la extracción de la APX y la MDHAR, se añadió ascorbato a una concentración de 10 mM al tampón de extracción (Polle y cols., 1990). Para la determinación de las actividades SOD, APX y MDHAR los sobrenadantes fueron purificados a través de microcolumnas PD SpinTrap G-25 (Sephadex) previamente equilibradas con tampón fosfato 20 mM pH 7.8 para la SOD y tampón fosfato 0,5 M pH 7 con ascorbato 1 mM para la APX y la MDHAR.

El método para la determinación de la actividad **SOD** (EC 1.15.1.1) se basó en la capacidad de la SOD de inhibir la reducción del citocromo C generada por radicales superóxido producidos por el sistema xantina-xantina oxidasa

(McCord y Fridovich, 1969). Una mezcla de reacción sin extracto vegetal se usó como control. El volumen de solución de xantina oxidasa (XOD) añadido se ajustó a fin de provocar un cambio de absorción de aproximadamente 0,025 unidades de absorbancia por minuto a 550 nm en el control. La mezcla de reacción (300 µl) estaba compuesta por tampón fosfato 50 mM (pH 7,8), EDTA 0,1 mM, xantina 0,82 mM, citocromo C oxidado 14 µM, el volumen ajustado de XOD y 20 µl de extracto vegetal. Una unidad de actividad se definió como la cantidad de SOD requerida para disminuir la tasa de reducción del citocromo C en un 50% a 25 ºC.

El método para la determinación de la actividad **APX** (EC 1.11.1.11) se basó en la monitorización de la desaparición de ascorbato a 290 nm y 25 ºC debido a la presencia de peróxido de hidrógeno (Asada, 1984). La mezcla de reacción (300 µl) estaba compuesta por tampón fosfato 50 mM (pH 7), ascorbato 0,8 mM, H_2O_2 2 mM y 20 µl de extracto vegetal. La oxidación no enzimática del ascorbato y la posible presencia de actividad ascorbato oxidasa se tuvieron en cuenta para realizar las oportunas correcciones. La actividad APX se expresó en moles de ascorbato metabolizados por minuto y mg de proteína. La cantidad de ascobato se calculó a partir de una curva estándar de ascorbato.

La actividad **MDHAR** (EC 1.6.5.4) se determinó mediante la monitorización de la oxidación de NADH a 340 nm y 25 ºC en presencia de ascorbato y ascorbato oxidasa (Borraccino et al., 1989). La mezcla de reacción (300 µl) se compuso de tampón tricina 110 mM (pH 8), ascorbato 0,88 mM, NADH 0,22 mM, 1,67 U ml^{-1} de ascorbato oxidasa y 20 µl de extracto vegetal. La oxidación espontánea del NADH y el consumo de esta coenzima en otras reacciones se tuvieron en cuenta para realizar las oportunas correcciones. La actividad MDHAR se expresó en moles de NADH oxidados por minuto y mg de proteína. La cantidad de NADH se calculó a partir de una curva estándar de NADH.

La actividad **glutation reductasa** (GR, EC 1.6.4.2) se determinó mediante la monitorización de la oxidación de NADPH a 340 nm y 25 ºC en la presencia de

glutation oxidado (GSSG) (Wingsle y Hällgren, 1993). La mezcla de reacción (300 µl) se componía de tampón HEPES 0,5 mM (pH 8), EDTA 0,5 mM, NADPH 125 µM, GSSG 0,5 mM y 30 µl de extracto vegetal. La oxidación espontánea del NADPH y el consumo de esta coenzima en otras reacciones se tuvieron en cuenta para realizar las correcciones oportunas. La actividad GR se expresó como moles de NADPH oxidados por minuto y mg de proteína. La cantidad de NADPH agotado se calculó a partir de una curva estándar de NADPH.

En la determinación de todas las actividades enzimáticas se emplearon microplacas de 96 pocillos para visible o UV, según la longitud de onda de medida requerida, y los espectrofotómetros Powerwave X 340 - BioTek Instruments, Inc. (para visible) y Multiskan Spectrum - Thermo Electron Corporation (para UV).

3.7.3. Pigmentos y tocoferoles

Las muestras vegetales se conservaron en oscuridad durante 16 horas para simular un pre-dawn. Posteriormente, se congelaron a -80 ºC durante un máximo de 3 meses hasta su análisis.

Para la determinación de pigmentos fotosintéticos (carotenoides y clorofilas) y tocoferoles se emplearon muestras de 25 mg (para *F. rubra*) o 40 mg (para *L. minor*) de peso fresco. La extracción se llevó a cabo en condiciones de iluminación tenue con morteros previamente enfriados con nitrógeno líquido (para *F. rubra*) o con un homogeneizador de tejidos con una velocidad de 10.000 rpm (Tissue tearor Bisopec Products, Inc. para *L. minor*) y 1,5 ml de acetona (100%) que contenía 0,5 g l^{-1} de $CaCO_3$ para evitar trazas ácidas que pudiesen alterar la composición pigmentaria de la muestra. El homogenado se centrifugó a 16.100 g durante 20 min a 4 ºC y el sobrenadante se filtró a través de filtros de jeringa de PTFE de 0,2 µm.

Los pigmentos y tocoferoles se determinaron simultáneamente según el método descrito por García-Plazaola y Becerril (1999) con las modificaciones de García-Plazaola y Becerril (2001).

Los tiempos de retención y coeficientes de conversión de los pigmentos se exponen en la tabla 3.4 y en la figura 3.5 aparece representado un cromatograma de *Lemna minor*.

Tabla 3.4. Tiempo de retención, longitud de onda de absorción máxima y coeficiente de conversión para clorofilas y carotenoides.

Pigmento	**Tiempo de retención (min)**	**λ max (nm)**	**Coeficiente de Conversión (10^{-4} pmol μV^{-1} s^{-1})**
Neoxantina	8,2	437,466	1,85
Violaxantina	9,3	441,471	1,48
Luteína epóxido	10,2	441,471	1,52
Anteraxantina	10,5	446,476	1,54
Luteína	11,4	446,476	1,47
Zeaxantina	11,6	451,481	1,63
Clorofila b	12,9	469,656	3,72
Clorofila a	13,9	435,666	5,34
α-Caroteno	15,6	446,471	1,41
β-Caroteno	15,8	451,480	1,72

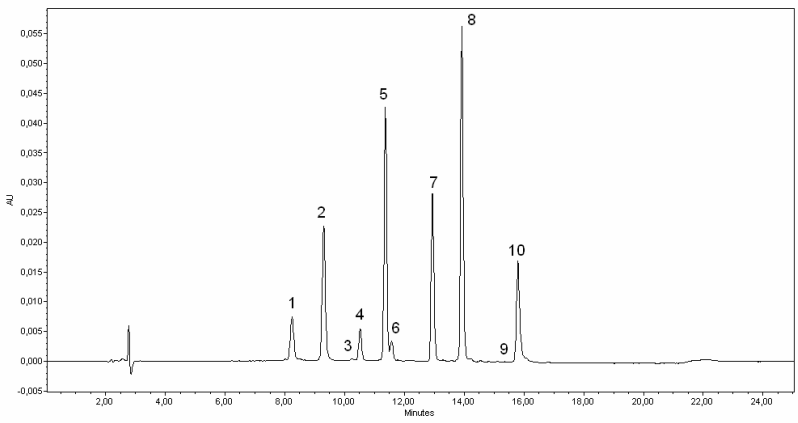

Fig. 3.5. Cromatograma de un extracto de *Lemna minor* a 445 nm. (1) Neoxantina, (2) Violoxantina, (3) Luteína epóxido, (4) Anteraxantina, (5) Luteína, (6) Zeaxantina, (7) Clorofila b, (8) Clorofila a, (9) α-caroteno, (10) β-caroteno. AU: unidades de absorbancia.

Los tiempos de retención, la longitudes de onda de excitación y emisión máximas y los factores de conversión de los distintos isómeros del tocoferol y de la feofitina se exponen en la tabla 3.5, mientras que en la figura 3.6 aparece representado un cromatograma de fluorescencia de *L. minor*.

Tabla 3.5. Tiempo de retención, longitudes de onda de excitación y emisión máximas y coeficiente de conversión para clorofilas y carotenoides.

Compuesto	**Tiempo de retención (min)**	**λ Excitación (nm)**	**λ Emisión (nm)**	**Coeficiente de conversión (10^{-4} pmol μV^{-1} s^{-1})**
δ-Tocoferol	11,4	290	340	6,51
β+γ-Tocoferol	12,7	290	340	3,8
α-Tocoferol	13,3	290	340	9
Feofitina	16,5	413	661	1,14

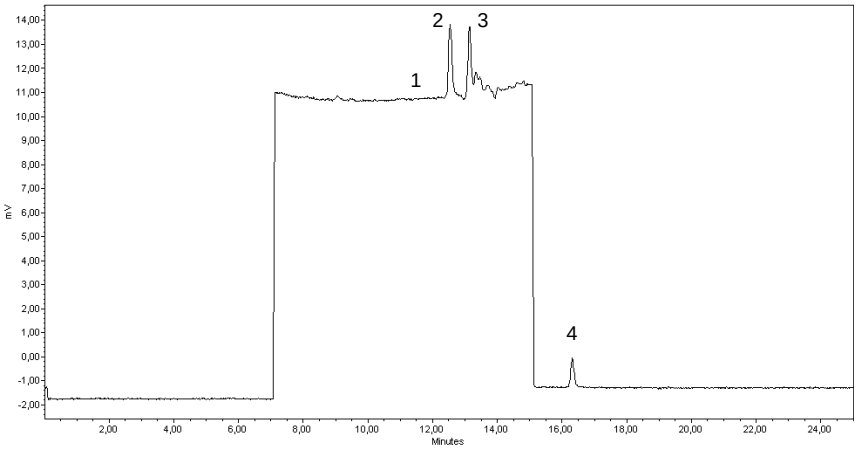

Fig. 3.6. Cromatograma de fluorescencia de un extracto de *L. minor*. (1) Protoclorofilida, (2) δ-tocoferol, (2) β+γ-Tocoferol, (3) α-Tocoferol, (4) Feofitina. mV: milivoltios.

3.7.4. Concentración de δ-ALA y actividad enzimática ALAD en L. minor

Las muestras empleadas se mantuvieron congeladas a -80 ºC durante un máximo de 6 semanas hasta su análisis.

Para la determinación de la concentración de **ácido δ-aminolevulínico** (δ-ALA) se adaptó el método de Becerril y cols. (1992). Se extrajeron 200 mg de peso fresco de colonias de *L. minor* con 2 ml de tricloroacético (TCA) al 4% (p/v) en un mortero previamente enfriado con nitrógeno líquido y bajo iluminación tenue. Se centrifugó el homogenado durante 20 min a 20 ºC y 16.100 g. A continuación, se añadieron 480 µl de acetato sódico 1 M y 30 µl de acetilacetona a 1 ml del sobrenadante y se calentó la mezcla a 100 ºC durante 10 min en un baño termostático. Tras dejar enfriar las muestras, se mezclaron volúmenes iguales de la muestra y de reactivo Ehrlich modificado (Becerril y cols., 1992) (500 mg de dimetilaminobenzaldehído, 5 ml de $HgCl_2$ en ácido acético glacial (1,5% p/v), 5 ml de $HClO_4$ al 70% y ácido acético glacial hasta completar 25 ml) y

se midió la absorbancia espectrofotométricamente (Multiskan Spectrum - Thermo Electron Corporation) en cubetas de vidrio a 555 nm tras 10 min. La concentración de δ-ALA se expresó en nmol de δ-ALA por gramo de peso fresco.

El método de Naito y cols. (1980) fue usado para la extracción de la enzima **ácido δ-aminolevulínico dehidratasa** (ALAD) (EC 4.2.1.24). En un mortero enfriado con nitrógeno líquido, se extrajeron muestras de peso fresco conocido con tampón Tris-HCl 0,05 M que contenía 0,5 ml g^{-1} PF de ditiotreitol (DTT) 0,1 mM, en una proporción 1:4 (p/v). El homogenado se centrifugó durante 45 min a 4 ºC y 16.100 g. La actividad ALAD se determinó según el método de Schneider (1970) con ligeras modificaciones. Se añadieron 270 µl de tampón Tris-HCl 0,05 M (pH 8,2) que contenía DTT 0,1 mM, 16 µl de $MgCl_2$ 0,2 M y 54 µl de δ-ALA (1 mg ml^{-1}), el iniciador de la reacción, a 200 µl de sobrenadante. La mezcla se incubó durante 1 hora a 37 ºC en un baño termostático. La reacción se paró con 60 µl de TCA 3 M. Tras dejar enfriar las muestras, se centrifugaron a 2.000 g durante 10 min y se mezclaron volúmenes iguales de reactivo Ehrlich modificado (Becerril y cols., 1992) y muestra. Transcurridos 10 min, se determinó la absorbancia de las muestras espectrofotométricamente como se ha descrito anteriormente para la concentración de δ-ALA. La actividad del enzima ALAD se expresó en nmol de porfobilinógeno (PBG) por hora y mg de proteína.

La concentración de δ-ALA y la actividad de ALAD se calcularon a partir de curvas estándar de δ-ALA y PBG, respectivamente.

3.7.5. Determinación de proteínas

Para determinar la cantidad de proteína en tejidos vegetales extraídos en medios acuosos con Tritón X-100 al 2% (en la determinación de actividades enzimáticas antioxidantes) se empleó el método de Lowry y cols. (1951) con las modificaciones de Peterson (1983) que permitían eliminar las interferencias que el detergente empleado en la extracción pudiese producir. Estas medidas se

hicieron a partir de extractos congelados a -20 ºC durante un máximo de 7 días. Previamente, se comprobó que la congelación de dichos extractos no suponía una degradación del contenido proteico total de la muestra. Se añadieron 75 µl de desoxicolato sódico (DOC) al 1% p/v (en NaOH 0,1 M) a 500 µl de extracto vegetal, se agitó y transcurridos 5 minutos, se añadieron 100 µl de ácido tricloroacético al 50%. Se agitó de nuevo la mezcla, se mantuvo 5 min en hielo y se centrifugó durante 10 min a 20 ºC y 16.100 g. Tras la eliminación del sobrenadante, se añadieron 100 µl de DOC al 0,25 % p/v (en NaOH 0,1 M) a la pastilla resultante, se agitó, se calentó en estufa a 80 ºC durante 5 min, se enfrío en hielo durante otros 5 min y se dejó reposar la mezcla durante 10 min a temperatura ambiente. A continuación, se añadió 1 ml de reactivo D ($CuSO_4.5H_20$ al 1% p/v en agua desionizada, tartrato disódico dihidratado al 1% p/v en agua y Na_2CO_3 al 2% p/v en NaOH 0,1 M en la proporción 1:1:98) y se agitó. Por último, transcurridos 10 min, se añadieron 100 µl de reactivo Folin-Ciocalteu diluido en agua (1:1) y se mezcló inmediatamente. Tras 30 min en oscuridad, se centrifugaron las muestras durante 5 min a 20 ºC y 21.280 g y se determinó la absorbancia de las muestras espectrofotométricamente a 750 nm (Powerwave X 340 BioTek Instruments, Inc.). El contenido proteico se determinó a partir de la elaboración de una recta de calibrado con seroalbúmina bovina.

El contenido en proteína soluble medido en los extractos acuosos empleados para la determinación de la actividad enzimática ALAD se hizo de acuerdo al método de Bradford (1976), usando seroalbúmina bovina como estándar. Las muestras se conservaron en frío hasta su análisis durante un máximo de 2 horas. En caso necesario, los extractos se diluyeron en tampón de extracción, y se mezclaron posteriormente con una dilución (6:23 v/v) de un reactivo comercial (BIO-RAD Protein Assay) (1:29 v/v). Se dejó reaccionar la mezcla durante 10 min en oscuridad y a continuación se midió la absorbancia espectrofotométricamente a 595 nm.

3.8. Análisis estadístico y tratamiento de imágenes

Se comprobó la normalidad (prueba de Kolmogorov-Smirnov) y la homogeneidad de varianzas (pruebas C de Cochran) de cada uno de los conjuntos de datos a comparar. Cuando la hipótesis nula de ambas pruebas fue aceptada, la posible existencia de diferencias entre tratamientos fue estudiada por medio de una ANOVA de una vía, seguida de la prueba de rango múltiple Duncan ($p<0,05$) en el caso de que el análisis de varianzas revelase la existencia de diferencias entre medias. Aquellos conjuntos de datos de distribución normal pero con varianzas heterogéneas fueron comparados por medio de la prueba t de Student para varianzas no homogéneas. Por último, los datos que no satisfacían ninguno de los dos requisitos anteriormente expuestos fueron comparados por medio de pruebas no paramétricas (H de Kruskal-Wallis y/o Z de Kolmogorov-Smirnov).

Los coeficientes de correlación ρ de Spearman (prueba no paramétrica para conjuntos de datos de distribución no normal) y Pearson (prueba paramétrica para conjuntos de datos de distribución normal) fueron calculados para determinar si existía asociación lineal entre dos variables ($p<0,05$).

Previo a la aplicación de un análisis de componentes principales (PCA), se determinaron los índices de esfericidad de Bartlett y de Kaiser-Meyer-Olkin para comprobar la adecuación del PCA. Esta técnica permite reducir la dimensionalidad de un conjunto de datos ayudando a hallar las causas de la variabilidad de esos datos y ordenándolas.

Los análisis estadísticos se llevaron a cabo con el programa SPSS v15.0 (2006) y v19.0 (2010). La prueba C de Cochran se llevó a cabo en Excel y los PCA se realizaron con el programa Canoco v. 4.5 (2002). Los programas Photoshop Lightroom 4 v. 4.0 (2007) e ImageJ 1.46r se emplearon para el tratamiento de imágenes (cap. 6).

4. Chemical stabilization of metal contaminated mine soil: Early soil amendment interactions and their effects on biological and chemical parameters

En la crisis nace la inventiva, los descubrimientos y las grandes estrategias.

(Albert Einstein)

4. Chemical stabilization of metal contaminated mine soil: Early soil-amendment interactions and their effects on biological and chemical parameters

María A. Galende, José M. Becerril, María T. Gómez-Sagasti, Oihana Barrutia, Lur Epelde, Carlos Garbisu, Antonio Hernández

4.1. Abstract

Chemical stabilization is an in situ technology that uses amendments to reduce soil metal bioavailability. Our objective was to determine the effectiveness of different amendments [sheep manure-SHEEP, poultry litter-POULTRY, cow slurry-COW, paper mill sludge mixed with poultry litter-PAPER] for chemical stabilization of mine soils. We assessed the early effects of amendments, in sterilized and non-sterilized form, on soil properties and phytotoxicity. Microbial populations present in amendments did not modify substantially the soil microbial functional diversity, as reflected by Biolog EcoPlates™, except in PAPER-amended soils. Lettuce root elongation was an excellent indicator of metal bioavailability. SHEEP and PAPER amendments were particularly effective at reducing metal bioavailability, while POULTRY and COW led to higher values of microbial properties. Our results emphasize the importance of simultaneously monitoring phytotoxicity and soil properties to properly identify bottlenecks during amendment selection for chemical stabilization in terms of reduction in metal bioavailability and improvement in soil health.

4.2. Introduction

Soil supports plant, animal and microbial life by providing habitat and nutrients. Moreover, soil is the basis of agriculture and livestock, which supply food and fibre for human use. Regrettably, soil contamination is a most pressing environmental problem, currently affecting the functionality of the soil ecosystem worldwide. In this regard, in Europe, about 1 800 000 sites are potentially contaminated (EEA, 2007), many of them with heavy metals. Heavy metal contamination can be caused by mining and industrial activities, fossil fuel combustion, incineration, agricultural practices, etc., resulting in potential toxic effects for all biota. Most importantly, heavy metals are known to bioaccumulate and biomagnify in the food chain (Fränzle et al., 2007).

Traditional physicochemical methods for soil remediation, including electrokinetics, excavation and burial, pyrometallurgical separation, incineration, washing, and solidification are frequently expensive and often result in irreversible damage on soil structure, biology and, hence, fertility (Alkorta et al., 2010; Mulligan et al., 2001). Thus, in the last decades, more environmentally-friendly and cost-effective technologies, such as phytoremediation (the use of green plants to remove contaminants from the environment or to render them harmless) (Salt et al., 1998) and chemical stabilization, are being developed. Chemical stabilization refers to the use of organic and/or inorganic amendments to modify soil characteristics such as pH, organic matter (OM) content, cation exchange capacity, etc. (Alvarenga et al., 2009a; Guo et al., 2006), in order to reduce metal mobility, bioavailability (through chemical changes and immobilization processes) and, concomitantly, metal toxicity (Ruttens et al., 2006). Specifically, chemical stabilization can be applied to highly contaminated sites where other environmentally-sound alternatives, such as metal phytoextraction, are not feasible.

Amendments for chemical stabilization can provide essential nutrients for plant growth (Alvarenga et al., 2009a), improve soil physicochemical properties

(such as water- and nutrient-holding capacity) (Tordoff et al., 2000), and stimulate soil biological activity (Arienzo et al., 2004). In addition, the use of organic residues for chemical stabilization enables recycling of wastes from farming and livestock activities.

Organic amendments are increasingly being used in combination with plants during “aided phytostabilization” processes (Epelde et al., 2009a). But most chemical stabilization and aided phytostabilization studies do not deal with (1) early soil-amendment interactions responsible for the stabilization-induced changes in soil physicochemical and biological properties, or (2) the impact of microbial populations present in organic amendments on soil microbial properties. Nonetheless, these two aspects require in-depth studies in order to optimize the selection of effective amendments for aided phytostabilization or chemical stabilization.

Within the context of the chemical stabilization of a metal contaminated mine soil, the aim of this study was to monitor the early effects of four organic amendments [sheep manure (SHEEP), poultry litter (POULTRY), cow slurry (COW), paper mill sludge mixed with poultry litter (PAPER)] on soil physicochemical and microbial properties, as well as on *Lactuca sativa* root elongation, as indicators of soil health and metal phytotoxicity, respectively. Implications to optimize the selection of amendments for chemical stabilization, in terms of (1) reduction in metal bioavailability and phytotoxicity, (2) minimum impact of microbial populations present in amendments on soil microbial properties, and, finally, (3) improvement in soil health, are discussed.

4.3. Materials and methods

4.3.1. Soil and amendment characterization

Rhizosphere soil (i.e., the zone of soil directly influenced by plant roots) (Lynch, 1990) was collected from the top layer (0-20 cm) of a vegetated area located in an abandoned mine belonging to Biscay province, northern Spain (latitude 43º13', longitude 3º26') (Barrutia et al., 2011). Table 4.1 shows the physicochemical properties of this metal (Cd, Pb, Zn) contaminated mine soil. Soil pH was measured in a 1:5 (w/v) suspension of oven-dried (80 ºC) soil and 0.01 M $CaCl_2$ (ISO 10390, 2005). Pseudo-total metal (Cd, Pb, Zn) concentrations were determined by ICP-AES (Inductively Coupled Plasma Atomic Emission Spectroscopy – Horiba Jobin Yvon Mod. Activa) in aqua regia digested soil samples (McGrath and Cunliffe, 1985). The 0.01 M $CaCl_2$-extractable fraction of metals, as an indicator of metal bioavailability, was determined according to Houba et al. (2000). For the determination of water holding capacity, the soil was saturated with deionised water, which was then extracted at -33 kPa for 48 h using a pressure plate extractor (1500F1 SoilMoisture Equipment Corp.) with 1 bar-pressure plate cells. Soil moisture was determined gravimetrically (Klute, 1986). Soil texture, OM content, organic carbon, total carbonates, total nitrogen and total phosphorus were determined following official methods (MAPA, 1994).

Four organic amendments were used in this study: sheep manure (SHEEP), poultry litter (POULTRY), cow slurry (COW), and paper mill sludge mixed (2:1, v/v) with poultry litter (PAPER). Chemical characterization of amendments (Table 4.1) was carried out as described in MAPA (1994). Amendment sterilization was performed in an autoclave at 120 ºC for 20 min. In order to check the effectiveness of sterilization, extracts from both sterilized and non-sterilized amendments were made by adding 1 g of amendment to 100 ml of sterile deionised water, followed by agitation (orbital shaker at 150 rpm) for 1 h. Subsequently, 0.5 ml of each extract were inoculated in 50 ml of sterile liquid culture medium (0.5 g l^{-1} K_2HPO_4, 0.2 g l^{-1} $MgSO_4.7H_2O$, 0.1 g l^{-1} NaCl, 0.2 g l^{-1} peptone, 10 g l^{-1} glucose, 0.2 g l^{-1} yeast extract) and incubated for 7 days at room

temperature. Our sterilization treatment proved to be effective, as sterilized amendments did not lead to microbial growth (OD_{680}) in the liquid medium, as opposed to non-sterilized amendments.

Table 4.1. Characterization of mine soil and amendments. Values are expressed on a dry weight basis.

	SOIL	POULTRY	PAPER	SHEEP	COW
Texture	sandy loam	-	-	-	-
WHC (%)	48.6	-	-	-	-
pH	5.6	7.6	8.2	9.1	6.1
OM (%)	5.7	73.1	33.1	62.7	79.7
Oxidizable OM (%)	4.4	54.4	28.7	38.9	71.4
$CaCO_3$ (%)	4	15	40	14	5
C/N ratio	14.4	5.9	28.5	8.3	17.8
Total N (%)	0.23	5.31	0.58	2.71	2.33
Total P_2O_5 (%)	0.05	5.62	0.9	3.47	1.29
Total K_2O (%)	-	3.33	0.65	7.14	3.51
Cd (mg kg^{-1})	4.99	0.21	0.18	0.22	0.12
Pb (mg kg^{-1})	34 692	0.65	16.4	3.39	2.61
Zn (mg kg^{-1})	17 576	487	325	482	524

POULTRY: poultry litter.
PAPER: paper mill sludge + poultry litter.
SHEEP: sheep manure.
COW: cow slurry.
WHC: water holding capacity.
OM: organic matter.

4.3.2. Experimental design

A chemical stabilization microcosm study was carried out for approximately one month in a phytotron under controlled conditions (24/18 ºC, 60/70% relative humidity-RH, 14/10 h day/night photoperiod, and a photosynthetic photon flux density -PPFD- of 462 µmol photon m^{-2} s^{-1}). Eight treatments were prepared by mixing 2 mm-sieved moist soil (the equivalent of 800 g of oven-dried soil) with blender-ground sterilized or non-sterilized amendment. In order to add an equal

amount of OM (1.3%) in all treatments, the mine soil was mixed (dry weight/dry weight) with 2, 2, 1.5 and 3.7% SHEEP, POULTRY, COW and PAPER amendment, respectively. Non-amended mine soil was used as CONTROL. Treatments were conducted in quadruplicate in 1-liter pots perforated at the bottom. Soils were bottom-watered regularly with deionised water to maintain a 70-80% water holding capacity, and rearranged randomly in the phytotron every 2-3 days.

At the beginning of the experiment (day 0: immediately after amendment application), as well as 7, 14 and 33 days after amendment application, a core of 170 cm^3 of soil was extracted from the top to the bottom of each pot, sieved (2 mm), and stored at 4 ºC prior to the determination of soil microbial properties. Alternatively, for the determination of soil physicochemical properties and the root elongation bioassay, soil samples were oven-dried at 80 ºC.

4.3.3. Root elongation bioassay

Seeds of *Lactuca sativa* L. were germinated under controlled conditions (25 ºC, 50% RH, darkness) for 2 days on wet filter paper placed in Petri dishes. In the meantime, 10 g of oven-dried soil were placed in 8.5 cm diameter Petri dishes, hydrated to 80-85% water holding capacity, covered with filter paper, and maintained for 2 days at 25 ºC and 50% RH. Twenty-five seeds of *L. sativa* showing a 5-mm long emerged radicle were transferred into the soil-containing Petri dishes and incubated for 3 days (25 ºC, 50% RH, 14/10 h day/night photoperiod and a PPFD of 100 µmol photon m^{-2} s^{-1}). Finally, root elongation (mm) was determined.

4.3.4. Soil microbial properties

Soil basal respiration (R_B), an indicator of overall soil microbial activity, and substrate-induced respiration (SIR), a measure of the potentially active microbial biomass, were determined according to ISO 16072 (2002) and ISO 17155 (2002), respectively, as modified by Epelde et al. (2008a): for R_B, fresh soil samples (the equivalent of 30 g of oven-dried soil) were placed in airtight jars, together with vials containing 0.2-0.8 N NaOH, and incubated for 3 days at 30 ℃. The CO_2 produced during the incubation period was determined by titration of remaining NaOH with 0.1 N HCl. For SIR, 10 000 mg C kg^{-1} (as glucose) were added to soil samples. After 6 h of incubation, respiration rate was measured as in R_B. Metabolic quotients (Q_R) were calculated as the ratio between R_B and SIR values.

Community-Level Physiological Profiles (CLPP) from soil microorganisms were obtained with Biolog EcoPlates™ following Epelde et al. (2008a). Colour development was read at 595 nm using a microplate reader (Anthos Zenyth 3100). Average well colour development (AWCD), Shannon's diversity index ($H' = -\Sigma p_i \log_2 p_i$), and species richness (S = number of utilized substrates with an absorbance value >0.25; this value marked the beginning of the exponential phase in the Biolog EcoPlatesTM) were determined after 40 h of incubation, which corresponded to the time of maximal microbial growth in the Biolog EcoPlates™. The Shannon's diversity index was calculated considering absorbances at each well as equivalent to species abundance. The kinetics of colour development for each substrate (except for phenylethylamine and 2-hydroxybenzoic acid, which were not utilized under any of the studied treatments) was summarized into one single value, by calculating the Area Under the "absorbance-sampling time" Curve (AUC) from data at day 0, 7, 14 and 33, using the trapezoidal formula (Fig. 4.1). This parameter (AUC) was chosen as it integrates the temporal changes that might occur in the rate of colour development (i.e., the capacity to utilize a specific substrate) throughout the experiment (Guckert et al., 1996), thus providing a measure of the total amount of each substrate utilized during the whole experiment.

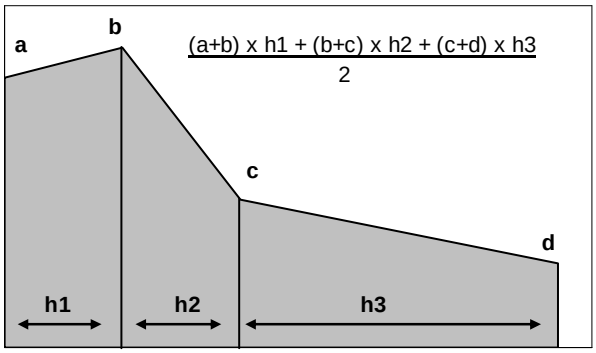

Fig. 4.1. Area Under the "absorbance-sampling time" Curve (AUC) calculation.

4.3.5. Statistical analysis

Every group of data was checked for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Cochran C test) and, when possible, differences between treatment means were analyzed by one-way ANOVA followed by Duncan test ($p<0.05$). Differences among data not satisfying assumptions for ANOVA were analysed using non-parametric tests (Kolmogorov-Smirnov Z or Kruskal-Wallis H). Pearson (parametric) and Spearman Rho (non-parametric) correlation coefficients were calculated to look for statistically significant ($p<0.05$) linear relationships between two variables. Those parameters which most differed between treatments (soil pH, metal bioavailability, R_B, SIR and root elongation) were chosen to perform a Principal Component Analysis (PCA). Previously, Bartlett sphericity and Kaiser-Meyer-Olkin indexes were calculated to check the adequacy of the PCA. All statistical analyses were carried out with the SPSS v. 15.0 software, except for PCA (Canoco v. 4.5 sotfware).

4.4. Results

4.4.1. Soil physicochemical parameters

The untreated soil, classified as Technosol (IUSS Working Group WRB, 2007), has a sandy loam texture, acidic pH, relatively high OM content, moderately low N content, and was highly contaminated with Cd, Pb and Zn (Table 4.1). Indeed, its pseudo-total metal concentrations largely exceeded maximum permissible levels established by the European Union (3 mg kg^{-1} for Cd, 300 mg kg^{-1} for Pb and Zn) (Directive 86/278/EC). On the other hand, pseudo-total metal concentrations in amendments were much lower than that observed in untreated soil (Table 4.1); in consequence, chemical stabilization treatments did not significantly alter soil pseudo-total metal concentrations (Table 4.2).

$CaCl_2$-extractable metal concentration in untreated soil was much higher than those found in amended soils (Fig. 4.2); in fact, at day 0**,** $CaCl_2$-extractable metal concentrations decreased in all treatments: on average, a 53-76, 54-77 and 45-71% reduction for Cd, Pb and Zn, respectively. This reduction was maintained throughout the experimental period, except for POULTRY treatment which, in general, showed an increase in $CaCl_2$-extractable metal concentrations from day 7 to day 33. The best results in terms of reduction in soil metal bioavailability (i.e., $CaCl_2$-extractable metal concentration) were obtained in SHEEP and PAPER treatments.

Table 4.2. Pseudo-total metal concentrations in soil before (CONTROL) and after amendment application (mean ± SE, n = 4). Values marked with the same letter are not significantly different ($p<0.05$). Units: mg kg^{-1} DW soil.

	Cd	Pb	Zn
CONTROL	4.99 ± 0.20 a	34 692 ± 7 824 a	17 576 ± 3 022 a
Sterilized POULTRY	5.17 ± 0.23 a	22 676 ± 2 869 a	17 079 ± 3 694 a
POULTRY	4.78 ± 0.01 a	18 636 ± 1 462 a	14 301 ± 1 826 a
Sterilized PAPER	5.19 ± 0.23 a	24 828 ± 4 623 a	13 623 ± 873 a
PAPER	5.58 ± 0.46 a	20 994 ± 3 108 a	12 399 ± 813 a
Sterilized SHEEP	4.99 ± 0.20 a	23 763 ± 2 551 a	14 442 ± 462 a
SHEEP	4.99 ± 0.20 a	20 247 ± 674 a	14 011 ± 1 256 a
Sterilized COW	5.39 ± 0.68 a	28 219 ± 4 752 a	18 220 ± 3 454 a
COW	5.18 ± 0.23 a	21 666 ± 2 001 a	16 629 ± 1 650 a

CONTROL: untreated.
POULTRY: poultry litter.
PAPER: paper mill sludge + poultry litter.
SHEEP: sheep manure.
COW: cow slurry.

Amendment application resulted in significantly higher values of soil pH throughout the experimental period (compared to CONTROL soil) (Fig. 4.3). Nonetheless, at day 0, POULTRY treatment showed the highest pH values among all treatments but, at day 33, pH values had decreased by 0.3-0.4 units. On the contrary, SHEEP, COW and PAPER treatments displayed a relatively constant pH value throughout the experiment. Interestingly, metal bioavailability was negatively correlated with pH ($p<0.01$, $R = -0.591$, -0.680 and -0.776 for Cd, Pb and Zn, respectively). This inverse relationship between metal bioavailability and pH was also observed in the PCA (Fig. 4.4)

Finally, in general, no significant differences in soil physicochemical parameters were observed between soils treated with sterilized versus non-sterilized amendments.

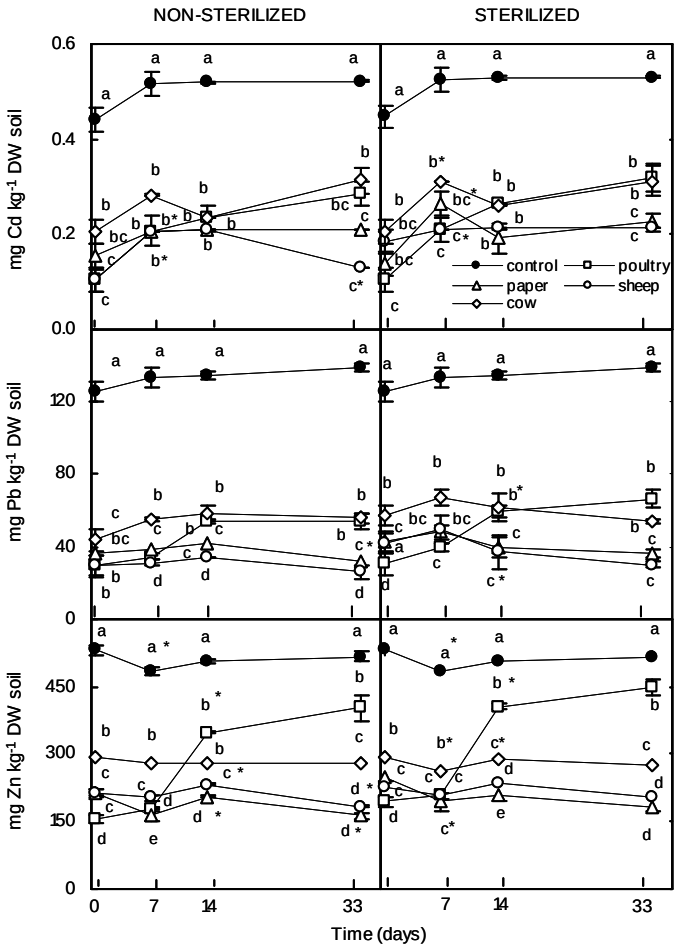

Fig. 4.2. $CaCl_2$-extractable metal concentrations in CONTROL and treated soils (mean ± SE, n = 4). Letters indicate significant ($p<0.05$) differences among treatments for each sampling time. Asterisks indicate significant ($p<0.05$) differences between a specific sampling time and the previous sampling time within each treatment. Control: untreated; Poultry: poultry litter; Paper: paper mill sludge + poultry litter; Sheep: sheep manure; Cow: cow slurry.

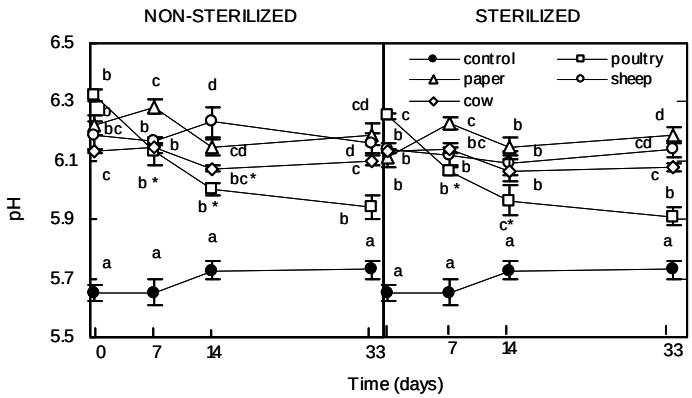

Fig. 4.3. Effect of treatments on soil pH (mean ± SE, n = 4). Letters indicate significant ($p<0.05$) differences among treatments for each sampling time. Asterisks indicate significant ($p<0.05$) differences between a specific sampling time and the previous sampling time within each treatment. Control: untreated; Poultry: poultry litter; Paper: paper mill sludge + poultry litter; Sheep: sheep manure; Cow: cow slurry.

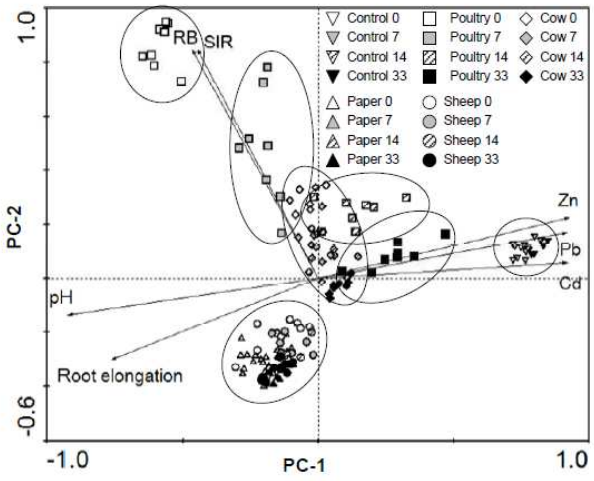

Fig. 4.4. Principal component analysis from values of soil chemical and microbial parameters. PC-1 and PC-2 account for 63 and 23% of variance, respectively. R_B: basal respiration; SIR: substrate-induced respiration; Zn, Pb and Cd: CaCl2-extractable metal concentrations. Control: untreated; Poultry: poultry litter; Paper: paper mill sludge + poultry litter; Sheep: sheep manure; Cow: cow slurry; 0: beginning of experiment; 7: 7th day; 14: 14th day; 33: 33rd day, end of experiment.

4.4.2. Root elongation bioassay

In general, values of L. sativa root elongation in untreated soil were lower than those observed in amended soils (Fig. 4.5). This parameter was negatively correlated with metal bioavailability ($p<0.01$, $R = -0.611$, -0.605 and -0.733 for Cd, Pb and Zn, respectively) and positively with soil pH ($p<0.01$, $R = 0.710$). At day 0, highest values of root elongation were found in POULTRY and PAPER treatments; nonetheless, this parameter clearly decreased over time under POULTRY treatment. On the other hand, under SHEEP treatment, values of root elongation were relatively constant throughout the experiment. In general, no significant differences were observed between soils treated with sterilized *versus* non-sterilized amendments.

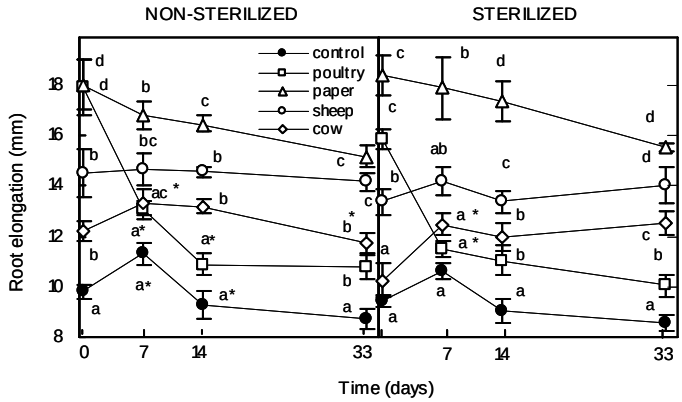

Fig. 4.5. Effect of treatments on Lactuca sativa root elongation (mean ± SE, n = 4). Letters indicate significant ($p<0.05$) differences among treatments for each sampling time. Asterisks indicate significant ($p<0.05$) differences between a specific sampling time and the previous sampling time within each treatment. Control: untreated; Poultry: poultry litter; Paper: paper mill sludge + poultry litter; Sheep: sheep manure; Cow: cow slurry.

4.4.3. Soil microbial properties

In untreated soil, values of R_B and SIR were very low throughout the experiment (Fig. 4.6). Basal respiration increased in all amended soils, as compared to CONTROL soil; by contrast, SIR increased only in POULTRY and COW treatments. Highest values of both parameters were obtained in POULTRY treatment. The stimulation of R_B in POULTRY and COW treatments was transient over time (a similar response was observed for SIR under POULTRY treatment).

At day 0, Q_R values were higher in PAPER and COW treatments than in untreated CONTROL (Fig. 4.6). Similarly, at day 7 and 14, Q_R values were higher in POULTRY than in untreated CONTROL. SHEEP treatment showed higher Q_R values than untreated CONTROL at day 33. In general, no differences were observed between soils treated with sterilized versus non-sterilized amendments.

In both untreated and treated soils, values of AWCD, S and H' decreased over time (Fig. 4.7). Under POULTRY treatment, higher values of these three Biolog^{TM} parameters were observed, as compared to untreated CONTROL. Values of AWCD, S and H' were positively ($p<0.01$) correlated with R_B and SIR. In general, no differences were observed between soils treated with sterilized versus non-sterilized amendments. Regarding the *t*otal amount of substrate utilized throughout the experiment, highest values in amended soils were observed for D-cellobiose, D-xylose, D-mannitol, glycogen and D,L-glycerol phosphate. In this respect, there was hardly any significant difference between soils treated with sterilized versus non-sterilized amendments, *except in PAPER-amended soils* (Fig. 4.8). PAPER treatment led to lower values of AUC than all the other treatments, including CONTROL soils. On the other hand, in terms of diversity (number of different types of utilized substrates) and quantity (number of utilized substrates), highest values were observed under POULTRY treatment (Fig. 4.8). Finally, when adding up AUC values for all substrates within each treatment, PAPER treatment was found to decrease AUC by 12%, while POULTRY increased it by 98%, as compared to untreated CONTROL (SHEEP and COW treatments resulted in a 19 and 30% increase, respectively).

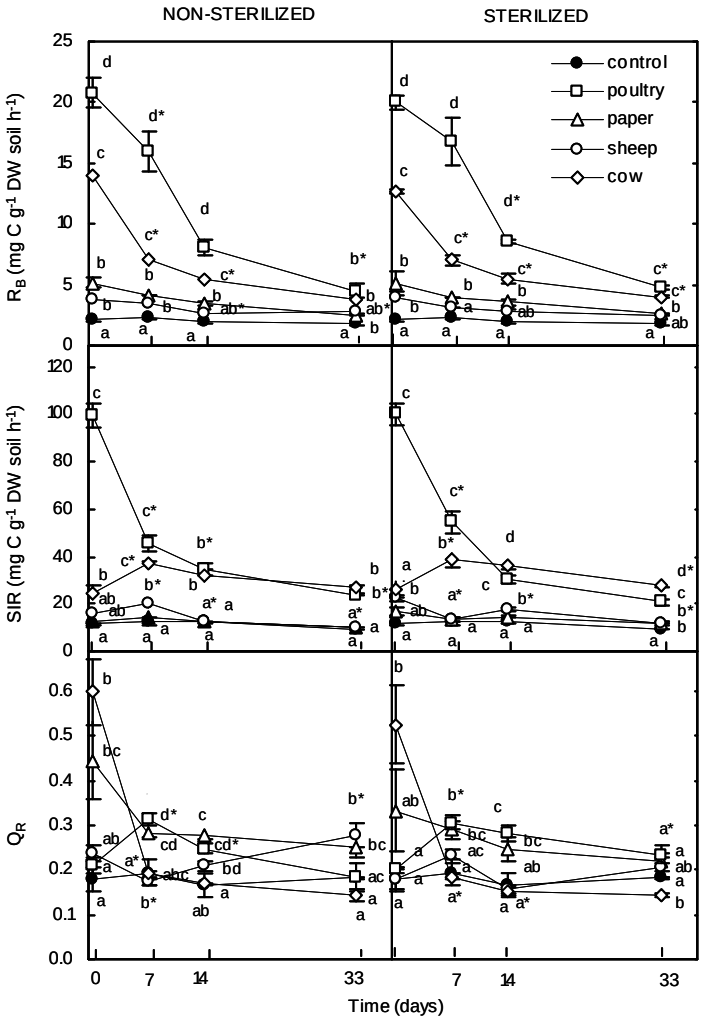

Fig. 4.6. Effect of treatments on basal respiration (R_B), substrate-induced respiration (SIR) and metabolic quotient (Q_R = R_B / SIR) (mean ± SE, n = 4). Letters indicate significant ($p<0.05$) differences among treatments for each sampling time. Asterisks indicate significant ($p<0.05$) differences between a specific sampling time and the previous sampling time within each treatment. For the determination of respiratory parameters, an incubation of 3 days was previously performed. Control: untreated; Poultry: poultry litter; Paper: paper mill sludge + poultry litter; Sheep: sheep manure; Cow: cow slurry.

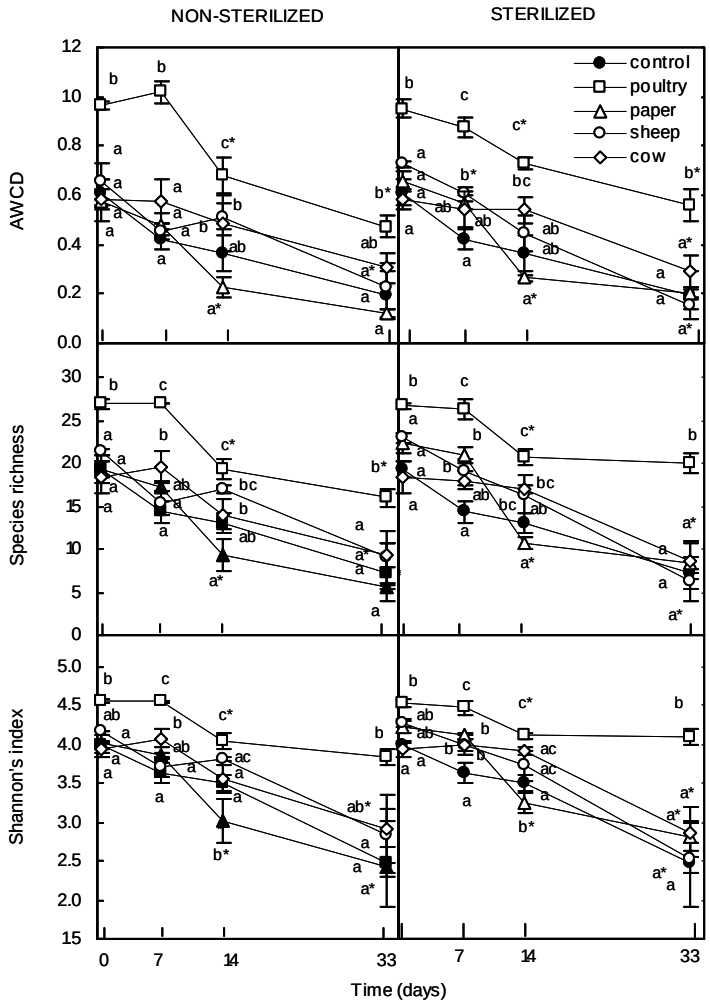

Fig. 4.7. Effect of treatments on Biolog EcoPlates™ data: AWCD, species richness (S) and Shannon´s diversity index (H') (mean ± SE, n = 4). Letters indicate significant ($p<0.05$) differences among treatments for each sampling time. Asterisks indicate significant ($p<0.05$) differences between a specific sampling time and the previous sampling time within each treatment. Control: untreated; Poultry: poultry litter; Paper: paper mill sludge + poultry litter; Sheep: sheep manure; Cow: cow slurry.

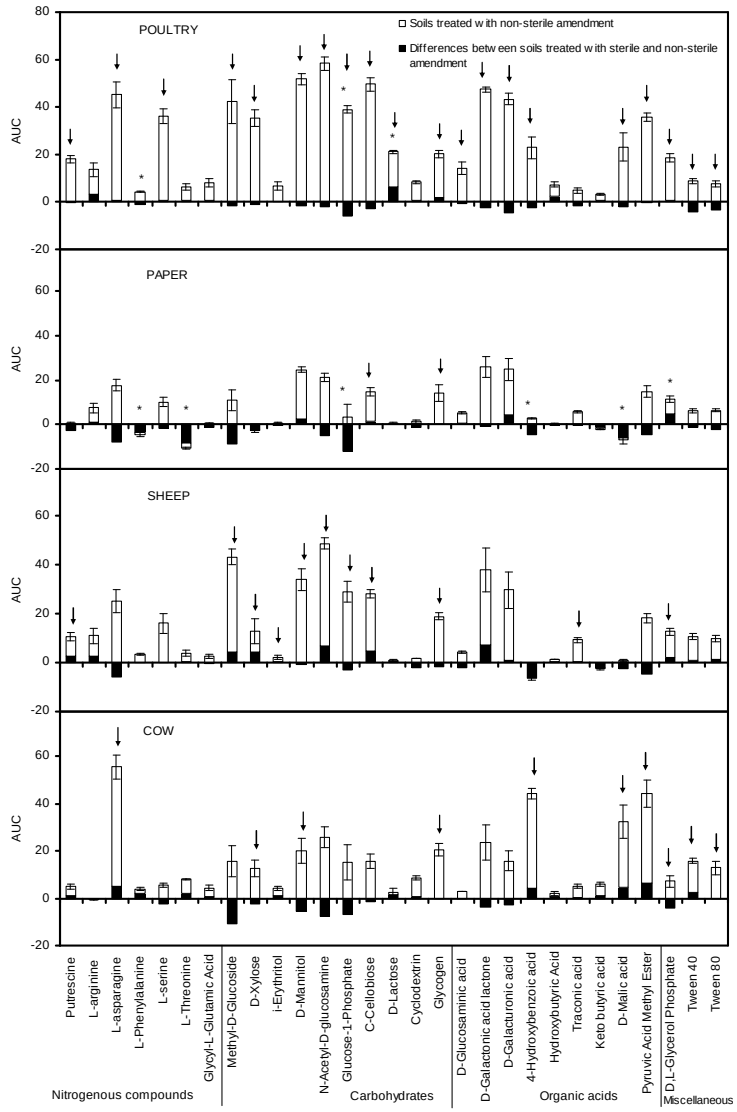

Fig. 4.8. Area Under the "absorbance-sampling time" Curve (AUC) for each substrate throughout the experiment (data from days 0, 7, 14 and 33) in non-sterilized amended soils, as well as differences in AUC values between soils treated with sterilized and non-sterilized amendments. Asterisks indicate significant ($p<0.05$) differences between soils treated with sterilized and non-sterilized amendments. Arrows indicate those substrates whose absorbance values were above 0.25 in at least 3 sampling times and whose AUC value was higher than that observed in CONTROL soil. Full length of the bar indicates AUC values of non-sterilized treatments; black areas represent the difference between soils treated with sterilized and non-sterilized amendments.

4.4.4. PCA results

According to the PCA (Fig. 4.4), a negative correlation was observed between metal bioavailability and soil pH; the same negative correlation was found between metal bioavailability and root elongation. In addition, the PCA revealed clear differences among treatments. Differences were also observed among sampling times under POULTRY treatment. In our PCA, the temporal variability of metal bioavailability and respiratory parameters (R_B, SIR) under POULTRY treatment was reflected. A temporal decrease in respiratory parameters was also reflected, but to a lesser extent, under COW treatment. On the other hand, SHEEP and PAPER treatments were more stable throughout the experiment in terms of reduction in metal bioavailability, increase in soil pH, and stimulation of root elongation.

4.5. Discussion

In most chemical stabilization and aided phytostabilization studies, the early effects of amendment application on unplanted soils have barely been investigated (Alvarenga et al., 2009c). However, shortly after amendment application, key changes in soil physicochemical and (micro)biological properties can occur with concomitant consequences for soil health. As a matter of fact, many aided phytostabilization studies start monitoring soil and plant parameters after a pre-determined period of amendment stabilization. However, the study of early soil-amendment interactions can help select the best amendment for ulterior remediation of metal contaminated soils.

In our study, immediately after amendment application, a decrease in metal bioavailability, related with an increase in soil pH, was observed (Figs. 4.1 and 4.2). Similar results were obtained by Calace et al. (2005), Gupta and Charles (1999) and Liu et al. (2009) in their studies with paper mill sludge, poultry litter and chicken manure compost, respectively. The negative correlation found here between soil pH and “bioavailable” metal concentrations confirms the well-known

influence of soil acidity in metal availability (Alvarenga et al., 2008a; Lee et al., 2009). Likewise, metal bioavailability can be reduced by precipitation with carbonates and phosphates present in amendments (Kumpiene et al., 2008; Walker et al., 2004), as well as by adsorption, complexation and redox reactions with both inorganic and organic matter (Adriano et al., 2004). The higher $CaCO_3$ content of PAPER amendment (Table 4.1) could explain, at least partly, the higher pH values shown under such treatment. Metal immobilization processes help reduce metal leaching, as well as metal absorption by organisms, thus minimizing their entrance into the food chain (Puschenreiter et al., 2005). In POULTRY treatment, the progressive increase in metal bioavailability, associated with a decreasing pH, could also be explained by the higher content of oxidizable OM present in the amendment (Table 4.1). Actually, organic matter degradation can induce the formation of organic and inorganic acids, contributing to soil pH reduction (McCauley et al., 2009). The rapid consumption of easily degradable OM might also explain the decrease in R_B found from day 0 to day 7 under POULTRY and COW treatments (Fig. 4.6).

Lactuca sativa root elongation negatively correlated with metal bioavailability and positively with soil pH. The root elongation bioassay is a sensitive, straightforward, cost-effective tool to estimate $CaCl_2$-extractable metal concentrations, and therefore could be used in phytoremediation monitoring programs. Escoto-Valerio et al. (2007) reported the sensitivity of L. sativa seed germination and root elongation to the presence of metals in soil-water solutions, and proposed those parameters as indicators of metal toxicity in contaminated soils.

From the beginning of the experiment (day 0), POULTRY and COW treatments showed higher values of R_B and SIR, suggesting a stimulation of the activity and biomass of soil microbial communities. An increase in R_B was also seen in PAPER and SHEEP treatments; however, these amended soils showed lower values of R_B and SIR than POULTRY- and COW-treated soils, despite the fact that “bioavailable” metal concentrations were lowest in PAPER- and SHEEP-treated soils, suggesting that the effect of amendments on soil microbial

properties depends, to a certain extent, on the nature of the amendment itself. As abovementioned, the amount of easily biodegradable OM in POULTRY and COW amendments was higher than in the other two amendments, which could explain the fast transient increase in microbial activity (R_B) and biomass (SIR). Bernal et al. (1998) reported that the presence of easily degradable OM in soils supplemented with mixtures of non-composted residues (sewage sludge, animal manures, city refuse, and industrial and plant residues) led to a high growth of soil microbial populations during the first three days of incubation. In our study, as abovementioned, the fast microbial consumption of this easily biodegradable OM, especially in POULTRY soils, could explain the progressive decrease in R_B and SIR observed over time. The reduction in microbial biomass (SIR) found in POULTRY soils was related to an increase in Pb and Zn bioavailability. Here it must be taken into consideration that SIR reflects the potential of soil zymogenous microorganisms (r-strategists) to degrade simple substrates (Anderson and Domsch, 1978). In fact, r-strategists are characterized by rapid growth when readily available nutrients are added to soil (Paul and Clark, 1996). On the other hand, in PAPER treatment, the high Q_R values suggest an amendment-induced ecophysiological stress on soil microbial communities.

The decrease in AWCD, S and H' observed over time in all soils, including untreated CONTROL (Fig. 4.7), could be due to our specific controlled experimental conditions, which appear to reduce the capacity of the soil microbial community to use different carbon sources (i.e., its functional diversity). In any event, higher values of these parameters were observed in POULTRY versus PAPER treatment. According to AUC values (Fig. 4.8), POULTRY treatment had the most positive effect on soil microbial functional diversity. By contrast, a loss of functional diversity was observed in PAPER-treated soil, compared to CONTROL soil: in this case, lower values of substrate utilization coincided with lower values of soil respiration. Sterilization of PAPER amendment caused a significant increase in the degradation of some specific substrates (L-phenylalanine, glycyl-L-glutamic acid, C-cellobiose, hydroxy butyric acid, D-malic acid and D,L-glycerol phosphate). In all treatments, highest values of substrate utilization were found

for carbohydrates, carboxylic acids and D,L-glycerol phosphate, most likely due to their being easily degradable carbon sources. CLPPs obtained with Biolog EcoPlates™ have proven to be a most useful tool for the assessment of (i) the suitability of organic amendments for chemical stabilization and (ii) their impact on soil microbial communities. In our study, the poultry litter in POULTRY and PAPER amendments came from different farms, which indicates that they might have a somewhat different composition. However, differences in soil microbial properties between POULTRY- and PAPER-treated soils might be due to the paper mill sludge having an inhibitory impact on Biolog EcoPlate™ parameters. In any case, PAPER was the most effective treatment in terms of reduction in metal bioavailability and stimulation of root elongation, at least partly due to an increase of heavy metal-sorbing sites (Battaglia et al., 2007).

The discrepancy between data on root elongation and soil microbial properties in response to amendments is not surprising, as root elongation is affected mainly by the presence of bioavailable metals, while soil microbial parameters are influenced not only by metal bioavailability but, most importantly, by other soil properties such as the quality and quantity of carbon sources, nutrient availability, etc. Indeed, in our mine soil, native soil microbial communities are adapted to the presence of high concentrations of toxic metals; thus, the short-term decrease in metal bioavailability appears much less relevant than the supply of easily degradable OM derived from amendment application. According to Baker et al. (2011), in metalliferous soils, available carbon is critical for the establishment and maintenance of healthy microbial populations. Our results highlight the importance of simultaneously monitoring both soil properties (with special emphasis on soil microbial parameters) and plant toxicity in chemical stabilization and aided phytostabilization studies, as the application of organic amendments can result in interactions not directly related with metal-induced toxic effects. Indeed, a comprehensive monitoring is highly useful for elucidating bottlenecks and limitations during the selection of the most appropriate amendments for the enhancement of soil functionality in chemical stabilization and aided phytostabilization studies.

On the other hand, in general, no significant differences were observed between soils treated with sterilized versus non-sterilized amendments, suggesting that microbial populations present in non-sterilized amendments did not substantially modify soil properties.

Finally, the use of organic wastes for the remediation of metal contaminated soils through chemical stabilization or aided phytostabilization helps reduce the amount of such wastes, while improving soil properties and reducing metal mobility, bioavailability and, hence, toxicity.

4.6. Conclusions

Chemical stabilization with organic amendments is a suitable, cost-effective alternative for the remediation of metal-contaminated mine soils. Sheep manure and paper mill sludge mixed with poultry litter were the best amendments in terms of reduction in metal bioavailability. In turn, poultry litter and cow slurry seemed most suitable for the improvement in soil health, as reflected by the values of the soil microbial parameters here determined. Microbial populations present in amendments did not modify substantially the soil microbial functional diversity, as reflected by Biolog EcoPlates™, except in PAPER-amended soils. Our results highlight the importance of simultaneously monitoring both soil properties (with special emphasis on soil microbial parameters) and plant toxicity (e.g., *L. sativa* root elongation as a sensitive, straightforward, cost-effective method) to properly assess the early effects of soil-amendment interactions during chemical stabilization and aided phytostabilization studies.

5. Beneficial effects of aided phytostabilization with Festuca rubra L. on Pb/Zn-polluted mine soils

Por el horizonte confuso y doliente
venía la noche preñada de estrellas.
Yo, como el barbudo mago de los cuentos,
sabía el lenguaje de flores y piedras.

Aprendí secretos de melancolía,
dichos por cipreses, ortigas y yedras;
supe del ensueño por boca del nardo,
canté con los lirios canciones serenas.

En el bosque antiguo, lleno de negrura,
todos me mostraban sus almas cual eran:
el pinar, borracho de aroma y sonido;
los olivos viejos, cargados de ciencia;
los álamos muertos, nidales de hormigas;
el musgo, nevado de blancas violetas.

(Federico García Lorca)

5. Beneficial effects of aided phytostabilization with *Festuca rubra* L. on Pb/Zn-polluted mine soil

María A. Galende, José M. Becerril, María T. Gómez-Sagasti, Oihana Barrutia, Carlos Garbisu, Antonio Hernández

5.1. Abstract

Aided phytostabilization is a technology that uses metal tolerant plants and amendments to reduce soil metal bioavailability and improve soil health. Our objective was to determine the effectiveness of amendment application [sheep manure (SHEEP), poultry litter (POULTRY), cow slurry (COW), paper mill sludge mixed with poultry litter (PAPER)] and growth of metallicolous *Festuca rubra* L. populations for aided phytostabilization of an abandoned Pb/Zn mine soil. To this end, we assessed the combined effects of amendments and plant growth on soil and plant properties. POULTRY was the best treatment in terms of plant performance and microbial biomass and activity, but resulted in higher levels of phytoavailable metals. SHEEP and PAPER were most effective at reducing metal bioavailability and, consequently, led to lower values of metal accumulation in plant tissues, thereby reducing the risk of metals entering the food chain. SHEEP was most effective at reducing metal bioavailability and plant metal content, as well as benefiting plant and microbial performance. The metallicolous population of *F. rubra,* combined with organic amendments, turned out to be an excellent candidate for aided phytostabilization. Our results suggest that organic fertilization is necessary for short term recovery of highly contaminated metalliferous soils, in spite of the growth of metallicolous species.

5.2. Introduction

Soil is the greatest reservoir of biodiversity on the planet (Crawford et al., 2005) and its interactions with microorganisms and plants are essential for the development of life at the surface of the Earth. Moreover, soil provides production, regulation and cultural services – the so-called ecosystem services – which benefit human populations in terms of resources and functions (Garbisu et al., 2011). Unfortunately, soil contamination is a worldwide environmental problem affecting soil health, i.e., its capacity to perform or function according to its potential (Hernández-Allica et al., 2006). Metals from natural or anthropogenic sources, such as mine wastes or industrial activities, are some of the main contaminants. Apart from metal contamination, mine soils have to deal with erosion, surface mobility, compaction and lack of organic matter (OM), nutrients, topsoil and soil-forming fine materials among others (Wong, 2003), which can inhibit soil-forming processes and plant growth.

Up to now, traditional expensive and environmentally damaging technologies have been used to cope with highly metal contaminated soils. An alternative method is aided phytostabilization, a more environmentally sound *in situ* technology using tolerant plants in combination with organic and/or inorganic amendments to reduce the bioavailability of toxic contaminants in soil. This strategy reduces soil erosion and metal leaching to groundwater, prevents dispersion of contaminated dust through wind and water (Vangronsveld et al., 2009), allows revegetation of contaminated sites and stimulates soil biological activity (Arienzo et al., 2004; Lambers et al., 2009). Furthermore, amendments improve soil physicochemical properties, such as pH and water holding capacity (Alvarenga et al., 2009b; Tordoff et al., 2000), reduce metal bioavailability and supply organic matter and nutrients to soils.

Tolerant flora to toxic metals is found in metalliferous soil ecosystems. The use of native plants in phytostabilization projects might ensure success, since they are adapted not only to tolerate metal contamination but also to other

adverse environmental factors typical of metalliferous sites (Ernst, 2005). *Festuca rubra* L., a graminoid commonly known as red fescue, is a pseudometallophyte growing both in pristine and metal contaminated soils. Previous studies of our group (Barrutia et al., 2011; Epelde et al., 2010) have identified this species in the mine site and have described it as a metalliferous population which hypertolerates metals based on exclusion strategies, i.e., restricting the entry of metals into the roots and/or their transport to the shoots (Baker, 1981).

Within the context of aided phytostabilization, the aim of this study was to assess the effects of four organic amendments [sheep manure (SHEEP), poultry litter (POULTRY), cow slurry (COW), paper mill sludge mixed with poultry litter (PAPER)] combined with a metallicolous population of *F. rubra* from a mining area on soil physicochemical and microbial properties, as well as on plant parameters and *Lactuca sativa* root elongation, as indicators of soil health, plant performance and metal phytotoxicity. We searched for the most effective amendment for aided phytostabilization of a Pb/Zn contaminated mine soil in terms of reduction in metal bioavailability and phytotoxicity and improvement in soil health and plant performance.

5.3. Materials and methods

5.3.1. Soil and amendment physicochemical characterization

A sandy-loam soil was collected from the top layer (0-20 cm) of a vegetated area located in an abandoned mine belonging to Biscay province, northern Spain (43º13' N, 3º26' W) (Barrutia et al., 2011). Table 5.1 shows the physicochemical properties of this metal (Pb, Zn) contaminated mine soil. Soil pH was measured in a 1:5 (w/v) suspension of oven-dried (80 ºC) soil and 0.01 M $CaCl_2$ (ISO 10390, 2005). Pseudo-total metal (Pb, Zn) concentrations were determined by ICP-AES (Inductively Coupled Plasma Atomic Emission Spectroscopy – Horiba Jobin Yvon Mod. Activa) in *aqua regia* digested soil samples (McGrath and Cunliffe, 1985).

The 0.01 M $CaCl_2$-extractable fraction of metals, as an indicator of metal bioavailability, was determined according to Houba et al. (2000). For the determination of water holding capacity, the soil was saturated with deionised water, which was then extracted at -33 kPa for 48 h using a pressure plate extractor (1500F1 SoilMoisture Equipment Corp.) with 1 bar-pressure plate cells. Soil moisture was determined gravimetrically (Klute, 1986). Soil texture, OM content, organic carbon, total carbonates, total nitrogen, total phosphorus and total potassium were determined according to official standards (MAPA, 1994).

Table 5.1. Characterization of amendments and mine soil. Parameters are expressed on a dry matter basis. Bioavailable concentrations of Pb and Zn are shown in parenthesis.

	POULTRY	PAPER	SHEEP	COW	Mine soil
pH	7.56	8.20	9.06	6.09	5.65
OM (%)	73	33	63	80	5.7
Carbonates (%)	15	40	14	5	4
C/N	5.94	28.5	8.32	17.8	14.4
N (%)	5.31	0.58	2.71	2.33	0.23
P_2O_5 (%)	5.62	0.9	3.47	1.29	0.056
K_2O (%)	3.33	0.65	7.14	3.51	-
Pb (mg kg^{-1})	0.65	16.4	3.39	2.61	34 692 (125)
Zn (mg kg^{-1})	487	325	482	524	17 576 (531)

POULTRY: poultry litter

PAPER: paper mill sludge with poultry litter

SHEEP: sheep manure

COW: cow slurry

OM: organic matter

C/N: carbon/nitrogen ratio

Four organic amendments were used in this study: sheep manure (SHEEP), poultry litter (POULTRY), cow slurry (COW), and paper mill sludge mixed (2:1, v/v) with poultry litter (PAPER). Chemical characterization of amendments (Table 5.1) was carried out as described in MAPA (1994).

5.3.2. Experimental design

Explants of *Festuca rubra* were collected from the contaminated mine. Plants were grown and propagated in an uncontaminated substrate made of peat moss under controlled greenhouse conditions (from 16 to 26 ºC) for 2 months prior to the experiment.

An aided phytostabilization microcosm study was carried out for 23 weeks in a phytotron under controlled environmental conditions (24/18 ºC, 60/70% relative humidity-RH, 14/10 h day/night photoperiod, and a photosynthetic photon flux density -PPFD- of 462 µmol photon m^{-2} s^{-1}). Four treatments were prepared by mixing 2 mm-sieved moist soil (450 g) with blender-ground amendment, and then allowed to stabilize in pots for 5 weeks. In order to add an equal OM content (1.3%) in all treatments, the mine soil was mixed (dry weight/dry weight) with 2, 2, 1.5 and 3.7% SHEEP, POULTRY, COW and PAPER amendments, respectively. After the stabilization period (5 weeks), three specimens of *F. rubra* (approximately 1.45 g of fresh weight per plant) were transplanted (Time 0) into the pots and allowed to grow for 18 weeks (Time 18 weeks). Non-amended mine soil was used as control. Treatments were conducted in quadruplicate in 0.5-liter pots perforated at the bottom. Soils were bottom-watered regularly with deionised water to maintain a 70-80% water holding capacity during the stabilization period and, subsequently, 100%, and rearranged randomly in the phytotron every 2-3 days.

Just before planting (Time 0) and at the end of the experiment (Time 18 weeks), soil samplles were sieved (2 mm) and stored at 4 ºC prior to the determination of soil microbial properties. Alternatively, for the determination of soil physicochemical properties and the root elongation bioassay, soil samples were oven-dried at 80 ºC.

5.3.3. Root elongation bioassay

Seeds of *Lactuca sativa* L. were germinated under controlled conditions (25 ºC, 50% RH, darkness) for 2 days on wet filter paper placed in Petri dishes. In the meantime, 10 g of oven-dried soil were placed in 8.5 cm diameter Petri dishes, hydrated to 80-85% water holding capacity, covered with filter paper, and maintained for 2 days at 25 ºC and 50% RH. Twenty-five seeds of *L. sativa* showing a 5-mm long emerged radicle were transferred into the soil-containing Petri dishes and incubated for 3 days (25 ºC, 50% RH, 14/10 h day/night photoperiod and a PPFD of 100 µmol photon m^{-2} s^{-1}). Finally, root elongation (mm) was determined.

5.3.4. Soils microbial parameters

Soil basal respiration (R_B), an indicator of overall soil microbial activity, and substrate-induced respiration (SIR), a measure of the potentially active microbial biomass, were determined according to ISO 16072 (2002) and ISO 17155 (2002), respectively, as modified by Epelde et al. (2008a): for R_B, fresh soil samples (the equivalent of 30 g of oven-dried soil) were placed in airtight jars, together with vials containing 0.2-0.8 N NaOH, and incubated for 3 days at 30 ºC. The CO_2 produced during the incubation period was determined by titration of remaining NaOH with 0.1 N HCl. For SIR, 10 000 mg C kg^{-1} (as glucose) were added to soil samples. After 6 h of incubation, respiration rate was measured as in R_B.

5.3.5. Plant parameters

Plants were harvested and separated into shoots and roots. Plant tissues were then washed with tap water to remove soil particles, rinse twice with deionised water and blotted dry with paper towels. After recording fresh weights, tissues were oven-dried at 80 ºC for 48 h to determine dry weights. Metal (Pb and

Zn) concentration in dry shoots and roots was determined according to Zhao et al. (1994) and metal phytoextraction (shoot metal concentration x shoot biomass) was calculated.

At the end of the experiment (Time 18 weeks), 0.025 g of leaf samples were collected before dawn to reduce the effects of diurnal variations in antioxidants and pigments and provide comparable predawn conditions. Then, leaves were frozen in liquid nitrogen and stored at -80 ºC until biochemical analysis. Chlorophylls were extracted in a pre-chilled mortar under dim light. Twenty five mg FW of frozen leaves were homogenized with 1.5 ml of acetone (containing 0.5 g l^{-1} CaCO3). The homogenate was centrifuged at 16,100 g for 20 min at 4 ºC and the supernatant was filtered through a 0.2 □m syringe filter. Chlorophylls were measured by reverse-phase HPLC (High Performance Liquid Chromatography) according to García-Plazaola and Becerril (1999) with the modifications described by García-Plazaola and Becerril (2001).

5.3.6. Statistical analysis

Each group of data was checked for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Cochran C test) and, when possible, differences between treatment means were analyzed by Student's t test ($p<0.05$) or one-way ANOVA followed by Duncan test ($p<0.05$). Differences among data not satisfying assumptions for ANOVA or Student's t test were analysed using non-parametric tests (Kolmogorov-Smirnov Z or Kruskal-Wallis H). Pearson (parametric) and Spearman Rho (non-parametric) correlation coefficients were calculated to look for statistically significant ($p<0.05$) linear relationships between two variables. Soil and plant parameters, as well as root elongation, were used to perform a Principal Component Analysis (PCA). Previously, Bartlett sphericity and Kaiser-Meyer-Olkin indexes were calculated to check the adequacy of the PCA. All

statistical analyses were carried out with the SPSS v. 15.0 (2006) software, except for PCA (Canoco v. 4.5 sotfware, 2002).

5.4. Results and Discussion

5.4.1. Soil chemical parameters

In aided phytostabilization processes, amendments are incorporated to decrease the bioavailable fraction of metals and to reduce the environmental risk. Moreover, organic residues provide essential nutrients for plant growth and organic matter which improves microbial properties of soil (Epelde et al., 2009a) and soil physical properties, as water infiltration and water holding capacity (Adriano et al., 2004). In this study, we assess the efficiency on phytostabilization of four amendments (SHEEP, POULTRY, COW and PAPER) applied to a mine soil with slightly acidic pH, relatively high OM content and moderately low N content and contaminated with Pb and Zn (Table 5.1). Pseudo-total concentration of Zn and Pb in soil (Table 5.1) highly exceeded maximum levels established by the European Union (300 mg kg^{-1} for Zn and Pb) (Directive 86/278/EC). Pseudo-total metal content, by analysis of strong acid digestion of soils, give an assessment of the maximum potentially soluble or mobile content of metals (Ure and Davidson, 2002). Lead presented the highest concentration, although Zn was the most bioavailable metal (Table 5.1). Considering that metal concentration in amendments was much lower than in soil and that the quantity applied ranged between 1.5 and 3.7% on a dry weight basis (see Materials and Methods), the application of amendments did not significantly alter pseudo-total metal concentrations.

After 5 weeks of stabilization process (Time 0), all soils treated with amendments decreased $CaCl_2$-extractable metal concentration (i.e., bioavailable metal concentration) of Pb and Zn (Fig. 5.1). At this time, this reduction was more effective for PAPER and SHEEP treatments. COW and POULTRY treatments

showed intermediate values of bioavailable metal. After transplanting and throughout the experiment, some remarkable changes occurred mainly in Zn bioavailability. The presence of *F. rubra* hardly modified metal bioavailability in POULTRY and SHEEP treatments, increased metal bioavailability in CONTROL (non amended soil) and COW treatment, which was markedly reduced in PAPER treatment (Fig. 5.1). These values of $CaCl_2$-extractable metal were related with changes in soil pH (see Fig. 5.1). In fact, we observed a negative correlation between decreasing pH and metal bioavailability ($p<0.01$, $R = -0.845$ and -0.936 for Pb and Zn, respectively) probably due to the fact that the rise in pH facilitates the adsorption of metals onto the soil binding sites, thereby reducing the concentration in soil solution (Liu et al., 2009). According to Adriano et al. (2004), organic residues can immobilize metals through adsorption, complexation and redox reactions. Furthermore, metals can precipitate with carbonates or phosphorous compounds provided by the amendments (Kumpiene et al., 2008). In fact, the high content of $CaCO_3$ content of PAPER treatment (Table 5.1) could explain the reduction of metal bioavailability and the higher soil pH. Our study indicates that the interaction between plant and amendment in soil is crucial to determine the fate of metal bioavailability. The pH alteration by plants could be caused by unbalanced nutrient uptake ratios by *F. rubra.* A greater cation over anion uptake can result in H^+ efflux from roots involving rhizosphere acidification and vice versa (Hinsinger et al., 2003).

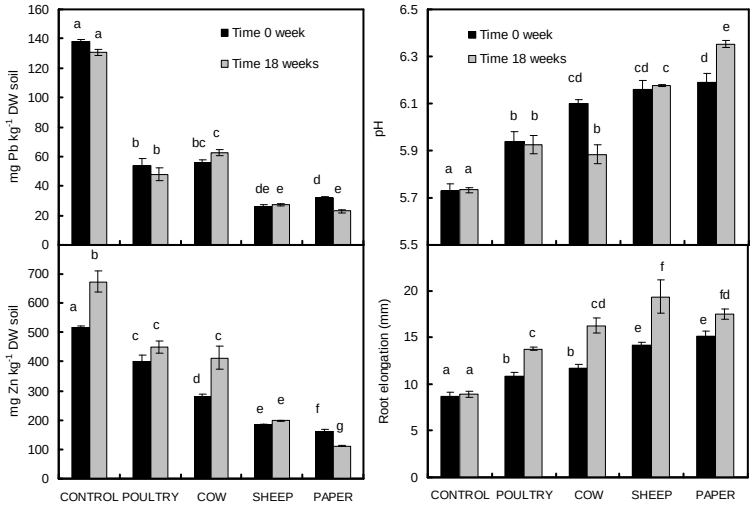

Fig. 5.1. Bioavailable Pb and Zn concentrations in soil, soil pH, and root elongation of *Lactuca sativa* at the beginning and at the end of the experiment (n = 4, mean ± SE). Different letters indicate statistically significant differences ($p<0.05$) among treatments POULTRY: poultry litter, COW: cow slurry, SHEEP: sheep manure, PAPER: paper mill sludge with poultry litter.

5.4.2. Root elongation bioassay

Toxicity tests with *Lactuca sativa* were used to assess soil ecotoxicity during the phytostabilization process. At the end of the stabilization period (Time 0), a significant enhancement of root length was recorded in amended soils (Fig. 5.1), especially in PAPER and SHEEP treatments, indicating a mitigation of soil toxicity that correlated with a decrease on metal bioavailability. The presence of *F. rubra* had an additional positive effect on root elongation of *L. sativa* seedlings exposed to amended soils, suggesting a beneficial effect of plant growth on soil health parameters. This beneficial effect through the phytostabilization process could be observed on treatments where $CaCl_2$-extractable metal concentration where maintained (POULTRY and SHEEP) or even increased (COW). However,

root elongation was not modified in CONTROL soil, indicating that reduction of ecotoxicity of soil during phytostabilization should rely on an appropriate combination of plants and amendments.

5.4.3. Soil basal and induced respiration

After 5 weeks of stabilization process (Time 0), all soils treated with amendments increased basal respiration (R_B), an indicator of soil microbial activity (Fig. 5.2). Through the phytostabilization study, all treatments (including CONTROL soils) significantly increased basal respiration rates. Proportionally, at the end of the study, the highest increases were observed in PAPER (81%) and SHEEP (41%) treatments, which presented the lowest rates at the beginning of the study. Interestingly, glucose-induced respiration (SIR), an estimation of microbial biomass of r-strategists (Dilly, 2006), was also significantly increased in PAPER and SHEEP treatments (Fig. 5.2). PAPER and SHEEP treatments presented the lowest metal bioavailability as well as the lowest rates of R_B and SIR, as compared to POULTRY and COW treatments. This suggests that other biotic and abiotic factors could influence native microbial populations. According to Grayston et al. (1998), biomass and activity of soil microorganisms are influenced not only by edaphic factors, such as soil type, nutrient and OM status, pH and moisture, but also by plant species and age. Although the quantity of OM added to the soil was the same for all the treatments, POULTRY and COW had more readily degradable organic substrates, which rapidly stimulated microorganism growth and activity in soil (Bernal et al., 1998; Ohm et al., 2011; Shi et al., 2005). On the other hand, benefits from plants could be more noticeable on soils presenting lower respiration rates. *Festuca rubra* could have benefited microbial populations by root exudation of nutrients, such as organic acids, sugars, lipids and proteins, and by providing additional surfaces for microbial colonization (Curl and Truelove, 1986; Tate, 1995) which support bacterial growth and activity. Our results indicated that in highly contaminated

soils, as mine soils, revegetation itself could not be enough, in a short term, to enhance biomass of microbial populations, but application of adequate amendments would contribute to increase significantly soil microbial biomass and activity.

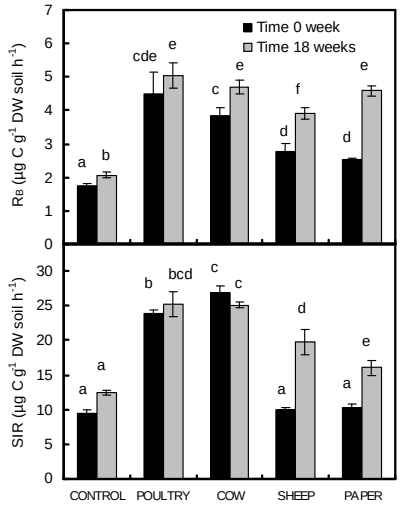

Fig. 5.2. Soil basal (R_B) and substrate-induced respirations (SIR) at the beginning and at the end of the experiment (n = 4, mean ± SE). Different letters indicate statistically significant differences ($p<0.05$) among treatments. POULTRY: poultry litter, COW: cow slurry, SHEEP: sheep manure, PAPER: paper mill sludge with poultry litter.

5.4.4. Plant parameters

In this study, we used a population of *F. rubra* from the mining area which was highly metal tolerant and a metal excluder plant (Barrutia et al., 2011; Epelde et al., 2010). At the end of the study, plants of *F. rubra* accumulated Pb and Zn in their roots and shoots (Fig. 5.3). Lead concentrations in roots and shoots ranged, among treatments and CONTROL, from 6,153 to 15,163 mg kg^{-1} DW and from 47 to 248 mg kg^{-1} DW, respectively. Due to the greater mobility of zinc, concentrations of Zn in shoots were higher than concentrations of Pb concentrations in roots were lower than in shoots, ranging, among treatments and

CONTROL, from 875 to 1,542 mg kg^{-1} DW and from 183 to 2,080 mg kg^{-1} DW for roots and shoots, respectively. Pb and Zn concentration in shoots and roots of *F. rubra* (Fig. 5.3) was decreased by amendment application reflecting the soil metal bioavailability (Fig. 5.1). In fact, we observed a significant correlation between soil metal bioavailability and metal concentration in shoots and roots for Zn ($p<0.01$, $R = 0.817$ and 0.640) and Pb ($p<0.01$, $R = 0.910$ and 0.706). Plants from PAPER and SHEEP treatments showed the lowest concentrations of Pb and Zn in shoots and lower phytoextraction levels of Pb and Zn during the study (18 weeks) compared to CONTROL plants (Fig. 5.3), reducing the risk of metal entrance into the food chain.

Overall, plant biomass (shoot and root) was enhanced by amendment application (Fig. 5.4). Plant biomass was positively correlated with R_B and SIR ($p<0.01$, R values were between 0.505 and 0.641), suggesting that amendments benefited not only plant growth, but also soil microorganism development, and that both mutually benefited each other, as it has been previously described (Epelde et al., 2009a, 2010).

The most effective treatment to increase plant biomass was POULTRY followed by SHEEP, COW and PAPER. Moreover, POULTRY was the only treatment which significantly increased shoot:root ratio. Since we add different amount of each amendments to mine soil in order to equal OM content in all the treatments, our results indicate that plant biomass responds to the input of nitrogen and phosphorous from the amendment itself (Table 5.1). When grown under high N supply, plants generally invest more biomass in shoots than in roots (van der Werf and Nagel, 1996). Nitrogen content influenced other plant parameters, such as chlorophyll content. Only plants from POULTRY-treated soils had significantly higher chlorophylls content (144%) than CONTROL (927 ± 99 nmol g^{-1} FW). Nitrogen is a structural element of chlorophyll; then, an increase in pigment content is an expected response to the supply of nitrogen (Ariz et al., 2010).

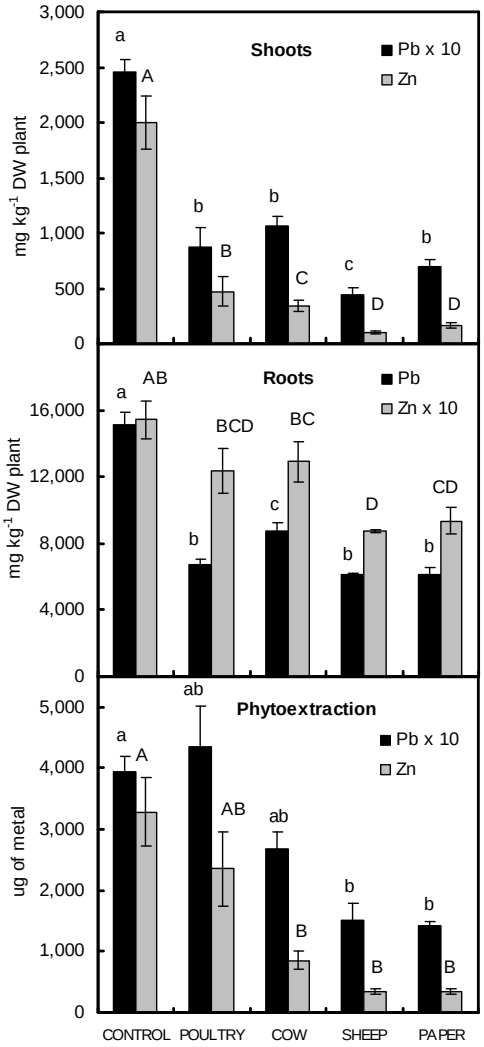

Fig. 5.3. Metal concentration in plant tissues, and metal phytoextraction of *F. rubra* at the end of the experiment (n = 4, mean ± SE). Different letters indicate statistically significant differences ($p<0.05$) among treatments. Shoot concentrations of Pb and Zn at time 0 were 2.8 ± 0.4 and 78 ± 6 mg kg^{-1}, respectively. Root concentrations of Pb and Zn at time 0 were 69.9 ± 6.5 and 59.0 ± 2.5 mg kg^{-1}, respectively. POULTRY: poultry litter, COW: cow slurry, SHEEP: sheep manure, PAPER: paper mill sludge with poultry litter.

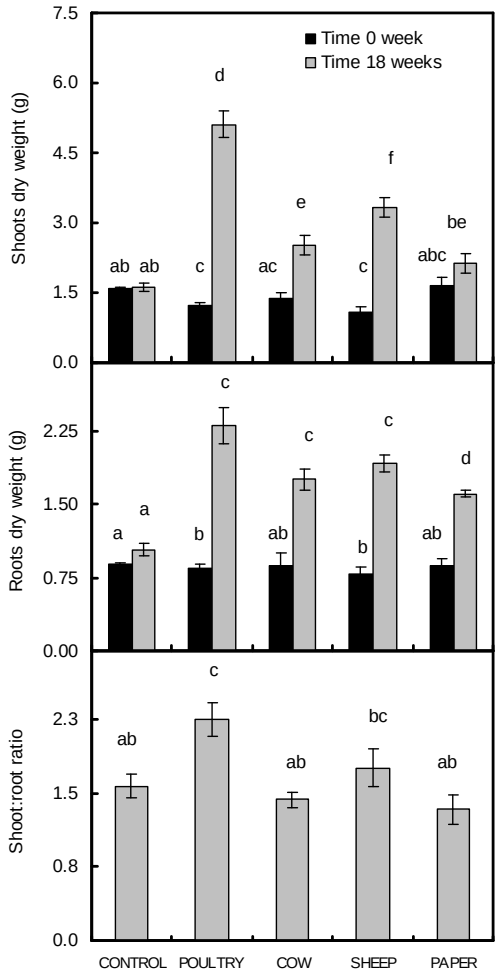

Fig. 5.4. Shoot and root dry weight of *F. rubra* at the beginning and at the end of the experiment, and shoot to root ratio of *F. rubra* at the end of the experiment (n = 4, mean ± SE). Different letters indicate statistically significant differences among treatments ($p<0.05$). POULTRY: poultry litter, COW: cow slurry, SHEEP: sheep manure, PAPER: paper mill sludge with poultry litter.

Although plants of *F. rubra* of POULTRY treatments seem to have a better performance (higher biomass and chlorophyll concentration), soil metal bioavailability and leaf metal content were highest under this treatment, indicating

that metal toxicity was not the main factor affecting the performance of this metallicolous plant population. Therefore, nutrient supply from amendments had a great relevance, conditioning plant growth and performance to a greater extent.

In a previous study, we observed the potential of inorganic fertilization to improve the physiological performance of other metallicolous species, such as, for instance, *Thlaspi caerulescens* grown in a highly contaminated soil (Hernández-Allica et al., 2006).

5.4.5. Principal Component Analysis

A PCA (using data from toxicity tests, as well as values of soil and plant parameters obtained at the end of the experiment) showed clear differences between amended and CONTROL soils, and also within amended soils (Fig. 5.5). On the one hand, CONTROL and treatments were separated along PC-1 (67% of variance): values of phytotoxicity, soil pH, metal bioavailability, Zn and Pb concentrations in shoots, Pb concentration in roots, R_B and root biomass are responsible for this separation. On the other hand, treatments were separated along PC-2 (19% of variance): in this case, plant biomass, SIR and soil pH were responsible for this separation.

Taking all data into consideration, POULTRY, SHEEP and PAPER treatments improved soil physicochemical conditions and biological activity of plants and microorganisms. Under POULTRY treatment, plants and microorganisms had a better performance but metal bioavailability and phytoavailability was higher. PAPER could be selected for phytostabilization where a reduction in metal bioavailability and plant metal content is required. Finally, SHEEP can be selected as an optimum treatment since it combines both a decrease in metal bioavailability and plant metal content with an acceptable performance in terms of benefiting plant and soil microorganisms.

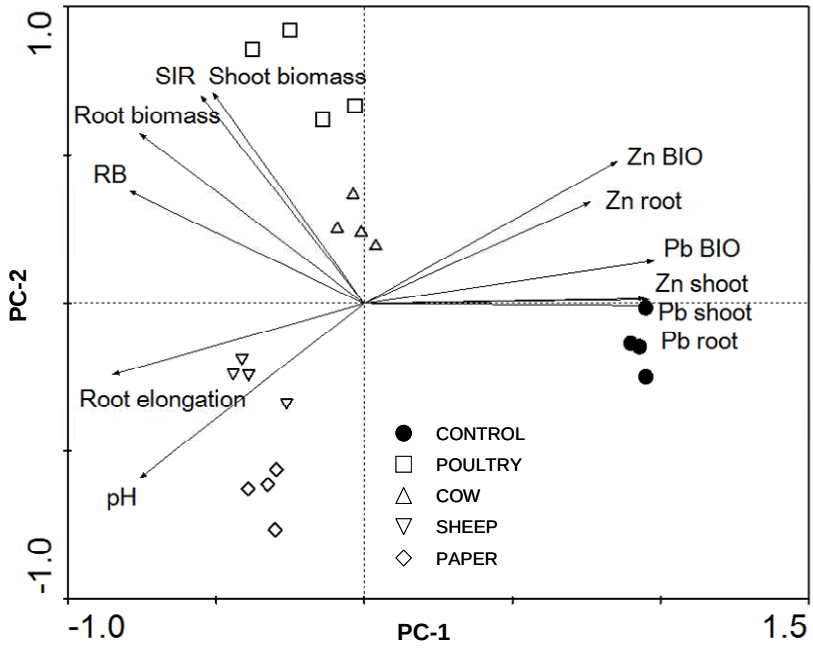

Fig. 5.5. Principal component analysis from values of soil chemical and microbial parameters. PC-1 and PC-2 account for 67 and 19% of variance, respectively. R_B: soil basal respiration, SIR: substrate-induced respiration, Bio: bioavailable, POULTRY: poultry litter, COW: cow slurry, SHEEP: sheep manure, PAPER: paper mill sludge with poultry litter.

Although we have previoulsy reported that this *F. rubra* population is a promising candidate for revegetation and phytostabilization of metalliferous soils (Barrutia et al. 2011; Epelde et al., 2010), this study presented here highlights that the application of organic amendments ensures a better plant establishment and, while improving soil microbiological properties and reducing ecotoxicity.

5.5. Conclusions

The application of organic amendments was responsible for an increase in soil pH, a decrease in metal bioavailability, and an increase in soil organic matter

and nutrient content, thus benefiting the development of plant and microbial populations. The presence of native *Festuca rubra* plants increased soil respiration and reduced soil ecotoxicity only in amended soils, suggesting that organic fertilization is necessary for short-term recovery of highly contaminated metalliferous soils. POULTRY was the best treatment in terms of increase plant performance and microbial activity and biomass, but resulted in higher levels of phytoavailable metals. SHEEP and PAPER were most effective at reducing soil metal bioavailability and, consequently, led to lower values of metal accumulation in plant tissues, thereby reducing the risk of metals entering the food chain. SHEEP was most effective at reducing metal bioavailability and plant metal content, as well as benefiting plant and microbial performance. The metallicolous population of *F. rubra,* combined with organic amendments, turned out to be an excellent candidate for aided phytostabilization. Furthermore, the use of farming or industrial organic by-products for phytostabilization would help reduce environmental risk associated with the disposal of manures, slurries and sludges. Our results indicate that the biological indicators studied here (plant toxicity test and microbial parameters) are useful tools for the evaluation of metal impact on ecosystem health. These biological indicators must be taken into consideration, together with soil physicochemical parameters, for a proper evaluation of soil health and the selection of the most appropriate amendments for aided phytostabilization.

6. Assessment of the efficiency of organic amendments for aided phytostabilization of vegetated and non-vegetated soil from a Pb/Zn contaminated mine

En las minas de Zinc que actualmente se esplotan en Lanestosa (Vizcaya) acaban de descubrirse objetos curiosos que demuestran que en tiempos muy remotos se han buscado allí los tesoros escondidos en el centro de la tierra. Se han presentado á la vista de los esploradores largas galerías y en las paredes de estas grandes candiles de barro que se cree serían para alumbrar por el día, porque como las galerías son muy bajas y los respiraderos que hacían de muy reducido diámetro debían necesitar luz. Se han hallado en estos mismos sitios varios instrumentos que por sus formas revelan ser muy antiguos y que los que usaban de ellos estaban poco adelantados en el arte de la esplotación. También se han sacado muchos trozos de madera sin labrar y cortados según se vé de una manera particular. El administrador de las minas que debe ser *sugeto curioso*, ha enviado á Madrid varios de los objetos notables por muchos conceptos.

J. de Granda. ***El Clamor Público***, viernes 2 de octubre de 1857.

6. Assessment of the efficiency of organic amendments for aided phytostabilization of vegetated and non-vegetated soil from a Pb-Zn contaminated mine

María A. Galende, José M. Becerril, Oihana Barrutia, Unai Artetxe, Carlos Garbisu, Antonio Hernández

6.1. Abstract

A small-scale field study was performed to assess the revegetation potential of native plant species and the effectiveness of organic amendments for aided phytostabilization of an abandoned Pb-Zn contaminated mine. Three amendments (cow slurry -COW-, poultry manure -POULTRY- and paper mill sludge mixed with poultry manure -PAPER-) were added to vegetated and non-vegetated areas within the abovementioned mine. Then, explants of native *Festuca rubra* were planted in non-vegetated areas. Two and six months later, the effects of treatments on vegetation and soil chemical and microbial parameters were studied. Amendment application to soil involved an input of organic matter and nutrients which led to a decrease in metal bioavailability, facilitating plant colonization and native plant growth, as well as stimulating soil microbial activity. COW and PAPER treatments were most successful at decreasing soil metal bioavailability in vegetated and initially non-vegetated areas, respectively. POULTRY was the best treatment in terms of plant growth and colonization in initially non-vegetated areas (up to 23.7% of vegetation cover). COW and PAPER treatments resulted in lower values of Pb and Zn concentration in *F. rubra* leaves from vegetated areas. COW was the best

treatment in terms of the stimulation of soil microorganisms in both vegetated and initially non-vegetated areas. Improvements in plant growth and soil microorganisms were higher in the most contaminated sites and in initially non-vegetated areas. The root elongation bioassay with *Lactuca sativa* proved to be a good bioindicator of soil metal bioavailability in phytostabilization processes under field conditions.

6.2. Introduction

Metals are inorganic non-biodegradable elements that can be present in soil naturally or as a consequence of anthropogenic activities such as mining or industry. Soils affected by mining activities suffer not only from elevated metals concentrations, but also from the absence of topsoil, water erosion, surface mobility, compaction, absence of soil-forming fine material, and shortage of essential nutrients and organic matter (Wong, 2003). All these constraints restrict plant growth and nutrient cycles through their adverse effect on soil microbiota (Epelde et al., 2009a). Moreover, toxic metals may enter into the food chain as a result of their absorption by soil microorganisms and plants.

In order to deal with soil metal contamination, several technologies have been traditionally used: excavation and transport to disposal facilities, incineration of the contaminated soil, on-site and off-site physicochemical treatments, etc. These technologies can negatively affect the soil ecosystem, with concomitant negative consequences for essential soil ecosystem services (i.e., the benefits people obtain from ecosystems (Millennium Ecosystem Assessment, 2005). In the last decades, environmentally-friendly, *in-situ* alternatives, such as phytoremediation (i.e., the use of green plants to remove contaminants from the environment or to render them harmless (Salt et al., 1998), have been developed in an attempt to revover soil health (i.e., the capacity of soil to perform its functions (Karlen et al., 1997). Among the different phytoremediation strategies,

phytostabilization, i.e. the use of plants to decrease soil contaminant bioavailability (Salt et al., 1998), is usually the option of choice in soils contaminated with high levels of toxic metals, such as mine soils. In mining areas, decontamination by phytoextraction is not feasible due to the long time required to reduce metal levels in soil. Moreover, phytostabilization allows the reutilization of farming and industrial wastes as amendments, which can: (i) reduce metal mobility in soil, (ii) decrease metal bioavailability to microorganisms, plants and animals, and hence toxicity, and (iii) increase nutrient input, thus stimulating biological activity of metalliferous soils. The establishment of a vegetative cover can reduce metal leaching to groundwater and the dispersion of metal-contaminated dust from bare or sparsely vegetated areas via wind or water erosion (Vangronsveld et al., 2009).

Many metalliferous sites, as mine areas, shelter a very diverse biodiversity of organisms which are adapted to tolerate not only the presence of metals but other adverse environmental factors as well. Native plant species from these areas can be used for revegetation or phytostabilization, since they are ecologically adapted to the adverse conditions of these sites. *Festuca rubra* L. (red fescue) has been previously described as an excluder pseudometallophyte and as the dominant plant species in the area of study (Barrutia et al., 2011; Epelde et al., 2010).

In spite of the large number of studies concerning aided phytostabilization (Alkorta et al., 2010; Bolan et al. 2011; Kumpiene et al., 2008) and the success of several pilot studies and field scale applications, it still remains not well understood how contaminants, soil, amendments, plant roots and microorganisms interact in the rhizosphere during phytostabilization processes (Epelde et al., 2012; Vangronsveld et al., 2009). This knowledge, as well as the need for reliable bioindicators of the recovery of soil health, is essential to perform a successful phytoremediation process from an economical, environmental and social point of view.

The present work is focused on soil health assessment of a phytostabilization process using native vegetation and organic amendments. We performed a small-scale field study in an abandoned Pb-Zn mine in order to: (i) assess the effectiveness of different organic amendments for the phytostabilization of vegetated and non-vegetated areas, (ii) assess the success of direct revegetation of bare soils with native plants, and (iii) assess the suitability of several chemical and biological indicators (plant and microbial parameters) to monitor the effectiveness of aided phytostabilization in terms of reduction on soil metal bioavaailability and soil health recovery.

6.3. Materials and Methods

6.3.1. Experimental design

A small-scale field study was carried out in an abandoned Pb-Zn mine located in the province of Biscay (Basque Country, Spain) (43º13' N; 3º26' W), whose characteristics have been described by **Barrutia et al. (2011)**. We selected two representative *Festuca rubra* L. vegetated areas (sites 1 and 2) and two representative non-vegetated areas (sites 3 and 4), showing high and low levels of bioavailable metals (Table 6.1). Organic amendments were superficially applied in 1 m^2 (1 m x 1 m) plots in order to add the equivalent to 150 kg of nitrogen (N) ha^{-1} of each amendment (Table 6.2). Each site had a control plot with no amendment. Explants from *Festuca rubra* were collected in the field and later grown in a peat moss substrate in a greenhouse, prior to their transplant in the field. After soil tillage, nine explants (approximately 1.45 g of fresh weight per plant) were transplanted to each plot in non-vegetated sites.

Table 6.1. Soils characterization of vegetated and non-vegetated sites. Values are expressed on a dry weight basis (mean ± SE, n = 3, except for pH and total and bioavailable concentration of metals where n = 4). Statistical analyses were performed for each parameter. Different letters indicate statistically significant differences among means (p<0.05).

	Vegetated sites		Non-vegetated sites	
	SITE 1	**SITE 2**	**SITE 3**	**SITE 4**
Sand (%)	55 ± 1 a	39 ± 3 b	50 ± 2 a	36 ± 2 b
Loam (%)	30 ± 2 a	39 ± 1 b	28 ± 2 a	34 ± 2 ab
Clay (%)	15 ± 2 a	22 ± 2 b	22 ± 1 b	30 ± 0 c
CEC (cmol kg^{-1})	8.3 ± 1.9 ab	5.4 ± 0.6 b	0.94 ± 0.02 c	4.0 ± 0.7 ab
pH	5.8 ± 0.0 a	5.9 ± 0.0 ab	6.1 ± 0.1 b	6.6 ± 0.1 c
OM (g kg^{-1})	175 ± 38 a	108 ± 10 a	8.2 ± 0.3 b	24 ± 3 c
Oxidable OM (g kg^{-1})	135 ± 29 a	83 ± 10 a	6.3 ± 0.2 b	18.5 ± 2.3 c
Nitrogen (g kg^{-1})	6.4 ± 1.5 a	4.9 ± 0.2 a	0.60 ± 0.03 b	1.4 ± 0.1 c
Ass P (g kg^{-1})	0.029 ± 0.012 ab	0.022 ± 0.002 a	0.017 ± 0.003 ab	0.012 ± 0.001 b
Assim K (g kg^{-1})	0.22 ± 0.05 a	0.11 ± 0.03 b	0.043 ± 0.016 b	0.047 ± 0.004 b
Assim Ca (g kg^{-1})	1.12 ± 0.26 a	0.70 ± 0.10 a	0.062 ± 0.004 b	0.56 ± 0.14 a
Assim Mg (g kg^{-1})	0.32 ± 0.08 ab	0.18 ± 0.02 a	0.052 ± 0.001 b	0.131 ± 0.009 a
CO_3^{2-} (g kg^{-1})	27 ± 3 a	40 ± 12 a	40 ± 6 a	47 ± 9 a
Total Pb (mg kg^{-1})	28383 ± 3328 a	46635 ± 3797 b	17364 ± 3966 a	52103 ± 4427 b
Total Zn (mg kg^{-1})	25774 ± 4833 a	71139 ± 11015 b	14918 ± 6396 a	87372 ± 4575 b
Pb bio (mg kg^{-1})	40 ± 4 a	65 ± 10 b	85 ± 4 b	23 ± 9 a
Zn bio (mg kg^{-1})	775 ± 48 a	1296 ± 66 b	377 ± 27 c	269 ± 49 c
Pb bio/total (%)	0.14 ± 0.02 a	0.14 ± 0.02 a	0.58 ± 0.13 a	0.05 ± 0.02 b
Zn bio/total (%)	3.33 ± 0.59 a	2.00 ± 0.39 ab	3.68 ± 0.95 a	0.32 ± 0.07 b

CEC: cation exchange capacity

OM: organic matter

Assim: assimilable

bio: bioavailable

bio/tot: ratio between the amount of bioavailable metal concentration and total metal concentration

Before amendment application (early spring of 2010) and 2 and 6 months later (late spring and end of summer, respectively), rhizosphere soil samples (10 cm depth) and aboveground plant specimens were sampled at each sampling site. Soil samples were sieved (2 mm). A portion of the sample was oven-dried at 38 ºC for the determination of soil physicochemical parameters and the plant

bioassay, while another portion was kept fresh at 4 ºC in the dark for the determination of soil microbial parameters. Plants were harvested, cleaned with a brush, rinsed with deionised water and oven-dried at 80 ºC for 48 h in order to determine dry weight and metal concentrations.

Table 6.2. Amendments characterization. Values are expressed on a dry weight basis. The amount of the different components added to soil is indicated in brackets (units: g m^{-2}, except for Pb and Zn: mg m^{-2}).

	COW	PAPER	POULTRY
EC (mS cm^{-1})	11.6	3.7	6.5
pH	5.9	7.7	7.9
OM (g kg^{-1})	860 (713)	510 (805)	73 (255)
Oxidable OM^e (g kg^{-1})	780 (647)	480 (757)	568 (197)
Humic acids (g kg^{-1})	20 (17)	18 (29)	36 (12)
Fulvic acids (g kg^{-1})	77 (64)	28 (45)	76 (26)
Nitrogen (g kg^{-1})	18 (15)	9.5 (15)	43 (15)
Phosphorus (P_2O_5) (g kg^{-1})	11 (9)	28 (44)	40 (14)
Potassium (K_2O) (g kg^{-1})	25 (21)	14 (22)	33 (12)
Calcium (CaO) (g kg^{-1})	24 (20)	182 (288)	93 (32)
Magnesium (MgO) (g kg^{-1})	7.4 (6.13)	12 (18.8)	8.5 (2.94)
$CaCO_3$ (g kg^{-1})	30 (25)	300 (474)	160 (55)
Pb tot (mg kg^{-1})	0.7 (0.5)	13.3 (21.0)	9.5 (3.3)
Zn tot (mg kg^{-1})	253 (210)	353 (558)	266 (92)

PAPER: paper mill sludge mixed with poultry manure

POULTRY: poultry manure

COW: cow slurry

EC: electrical conductivity

OM: organic matter; tot: total

6.3.2. Soil and amendment characterization

Table 6.1 shows the physicochemical characterization of the four studied soils (sites 1 to 4). Soil texture, cation exchange capacity, total and oxidable OM, total N, carbonate conntent, and assimilable phosphorous, potassium, calcium and magnesium concentrations were determined according to standard methods

(MAPA, 1994). Soil pH values were determined in a 0.01 M $CaCl_2$ solution (1:5, w/v) after vigorously shaking for 2-3 min (Rayment and Higginson, 1992; Jones, 2001), while Pb and Zn pseudo-total and bioavailable concentrations were measured by ICP-AES (Inductively Coupled Plasma Atomic Emission Spectroscopy) after *aqua regia* digestion (McGrath and Cunliffe, 1985) and 0.01 M $CaCl_2$ extraction (Houba et al., 2000), respectively. Pseudo-total metal content, by analysis of strong acid digestion of soils, give an assessment of the maximum potentially soluble or mobile content of metals (Ure and Davidson, 2002). For the determination of soil water holding capacity, a pressure plate extractor (1500F1 SoilMoisture Equipment Corp.) with 1 bar-pressure plate cells was used.

Three organic amendments were used in this study: cow slurry (COW), paper mill sludge mixed with poultry manure (2:1, v/v) (PAPER) and poultry manure (POULTRY). Chemical characterization of amendments (Table 6.2) was performed following standard methods (MAPA, 1994).

6.3.3. Root elongation bioassay

Seeds of *Lactuca sativa* L. were germinated under controlled conditions (18/25 ºC day/night, darkness) for 2 days on wet filter paper placed in Petri dishes. In the meantime, 10 g of oven-dried soil were placed in 8.5 cm diameter Petri dishes, hydrated to 80-85% water holding capacity, covered with filter paper, and maintained for 2 days at 18/25 ºC (day/night). Twenty-five seeds of *L. sativa* showing a 5-mm long emerged radicle were transferred into the soil-containing Petri dishes and incubated for 3 days (18/25 ºC day/night, 14/10 h day/night photoperiod and a PPFD of 100 µmol photon m^{-2} s^{-1}). Finally, root elongation (mm) was determined.

6.3.4. Soil microbial properties

Soil basal respiration (R_B) and substrate-induced respiration (SIR) were determined according to ISO 16072 (2002) and ISO 17155 (2002) standards, respectively, as described by Epelde et al., (2008b). For R_B, fresh soil samples (equivalent to 30 g of dry weight) were placed in airtight jars together with vials containing NaOH and incubated for 3 days at 30 ℃. The CO_2 produced during the incubation period was determined by titration of remaining NaOH with HCl. For SIR, 10,000 mg C kg^{-1} (as dissolved glucose) were added to the same soil samples. After 6 h of incubation, the respiration rate was measured as described for R_B.

β-glucosidase (EC 3.2.1.21), arylsulphatase (EC 3.1.6.1), acid phosphatase (EC 3.1.3.2) and urease (EC 3.5.1.5) activity were spectrophotometrically determined as described by Epelde et al. (2008a). Activity of fluorescein diacetate (FDA) hydrolysis was estimated according to García et al. (1993), Taylor et al. (2002) and Shaw and Burns (2006): 1 g of fresh soil, 4 ml of 0.1 M Tris (pH 7.6) and 50 µl of FDA solution (0.2% w/v in acetone) were mixed and incubated for 12 min at 25 ºC. The reaction was stopped with 4 ml of acetone and, after centrifugation (14,000 rpm, 5 min), absorbance was spectrophotometrically determined at 490 nm. The geometric mean of the values of all enzyme activities (Overall Enzyme Activity, OEA) was calculated according to formula [1].

$$OEA = \sqrt[5]{(\beta\text{-glucosidase} \times \text{arylsulphatase} \times \text{acid phosphatase} \times \text{urease} \times \text{FDA hydrolisis})} \quad [1]$$

Biolog EcoPlate™ was used for average well colour development (AWCD) determination according to Epelde et al. (2008b). Colour development was read at 595 nm using a microplate reader and AWCD was determined after 40 h of incubation, which corresponded to the time of maximal microbial growth in the Biolog EcoPlate™.

6.3.5. Plant parameters

Growth index of plant biomass in vegetated sites was calculated according to formulae [2]. Metal phytoextraction (MP) was measured according to formulae [3]. In order to determine vegetation cover in initially non-vegetated sites, Photoshop Lightroom 4 v. 4.0 (2007) and ImageJ 1.46r softwares were used for image treatment.

Growth index = Total biomass/Initial biomass [2]

MP = (Biomass at 2 months x Metal shoot concentration at 2 months)
+ (Biomass at 6 months x Metal shoot concentration at 6 months) [3]

6.3.6. Statistical analysis

ANOVA or Student's t-tests were performed to look for statistically significant differences among means ($p<0.05$). Previously, data normality was tested through the Kolmogorov-Smirnov test, while Cochran's C test was used for testing homogeneity of variance. Duncan test was performed when ANOVA results indicated differences among means ($p<0.05$). Linear regression coefficients (R^2) were calculated to determine relationship between variables ($p<0.05$). Data on soil chemical and microbial parameters, plant toxicity (root elongation bioassay) and plant biomass were used to perform a Principal Component Analysis (PCA). Bartlett sphericity and Kaiser-Meyer-Olkin indexes were calculated to check the adequacy of the PCA. All statistical analyses were carried out with the SPSS v. 15.0 (2006) software, except for PCA (Canoco v. 4.5 sotfware, 2002).

6.4. Results and Discussion

6.4.1. Physichochemical and biological characterization of the study area

The Pb/Zn mine is located in the temperate Atlantic Region of Spain. The climate is temperate, wet, with a mean annual rainfall of about 1,400 mm, and a mean annual temperature of 11-15 ºC (Euskalmet, 2010). The surrounding mining area includes open pits, waste rocks, tailing dams and side parts affected by mining subsidence. The mining area has been spontaneously revegetated in the last 30 years, after mining activities ceased in the late 1970s, and contains a wide range of metallicolous plant populations, including the hyperaccumulator *Thlaspi caerulescens* and several metal excluders such as *Ulex europeus*, *Agrostis capilaris* and *Festuca rubra* (Barrutia et al., 2011). *Festuca rubra* is the dominant plant species in this area and has been recommended for revegetation and phytostabilization of mine tailings (Epelde et al. 2010; Becerra-Castro et al., 2012).

We selected four sites: two areas vegetated mainly with *F. rubra* (SITES 1, 2) and two non-vegetated areas of (SITES 3, 4), showing high and low levels of bioavailable metals. As observed in many other mine tailings (Ye et al., 2002), our studied sites have a low mineral nutrient content and high levels of total and bioavailable metals (Table 6.1). Soils were deficient in essential plant macronutrients (N, P, K), which can explain the low vegetation cover and plant growth. Soils from vegetated sites presented higher nutrient mineral status and higher levels of OM (81-233 g kg^{-1}), as compared with non-vegetated soils (8-30 g kg^{-1}). The lack of OM in the latter location, which might be related with the absence of a plant cover (plant decomposition incorporates OM to the soil), could greatly influence the activity of soil microbial communities.

The main metals in the soil were Pb and Zn. Pseudo-total metal concentrations were highly heterogeneous among sites (Table 6.1) and exceeded official threshold values established by the Basque Government for ecosystem

protection (330 mg kg^{-1} and 840 mg kg^{-1} for Pb and Zn, respectively). Similarly, we determined soil $CaCl_2$-extractable metal concentration (i.e., metal bioavailability), as it is considered the metal fraction readily available to microorganisms and plants (Peijnenburg and Jager, 2003). Values of bioavailable Zn concentration and its percentage with respect to the pseudo-total concentration were higher (0.3-3.7%) than for Pb (0.05-0.6%) (Table 6.1), reflecting the higher mobility of Zn in soils. SITE 2 (vegetated) had the highest concentration of bioavailable metals, followed by SITE 1 (vegetated), SITE 3 (non-vegetated) and SITE 4 (non-vegetated). SITE 4 had the highest total concentrations of Pb and Zn in soil, but the lowest metal bioavailability (Table 1). According to Ross (1994), pH, OM (concentration and composition), phosphates, carbonates and clay content, cation exchange capacity, the presence of competing cations, etc. can enhance or limit metal sorption in soil. SITE 4 had a significant higher pH than the other sites and, as observed in previous studies (Wong et al., 2003; Galende et al., *in preparation*, Chapter 1 and 2), could be a relevant parameter to reduce metal bioavailability.

Regarding the initial biological characterization of soils, the values of all microbial properties were much higher in vegetated SITES 1 and 2 (i.e., basal respiration was 9.0 and 5.8 mg C kg^{-1} DW soil h^{-1}, respectively; overall enzyme activity was 334 and 161, respectively) as compared with non-vegetated SITES 3 and 4 (i.e., basal respiration was 1.1 and 3.1 mg C kg^{-1} DW soil h^{-1}, respectively; overall enzyme activity was 17 and 32, respectively). Soil ecotoxicity, measured through the root elongation bioassay, was highest in SITE 3. Considering all biological parameters, the affection trend was as follows: SITE 3 >SITE 2>SITE 4>SITE 1. Although vegetated sites presented higher metal bioavailable fractions, the biological parameters were much lower in non-vegetated sites (especially SITE 3). The presence of vegetation had a beneficial effect on soil biological properties (Hernández-Allica et al., 2006), that could account for the observed results at the initial conditions.

6.4.2. Effect of amendments on soil chemical parameters

The application of organic amendments to mine soils for aided phytostabilization aims at immobilizing metals, improving soil physicochemical characteristics, supplying nutrients for plant growth, and increasing soil biological activity (Alkorta et al., 2010). In this study, we tested the efficiency of three livestock/industrial byproducts for aided phytostabilization. All amendments were applied in order to add 150 kg of N ha^{-1}, and due to their different chemical composition, COW- and PAPER-treated plots had higher levels of OM and K^+ than POULTRY-treated plots. PAPER-treated plots had much higher levels of Ca^{2+} and $CaCO_3$ (Table 6.2). The incorporation of those nutrients from amendments to soils could contribute to overcome the low mineral nutrient content of the mine soils, especially in non-vegetated (SITES 3, 4) which presented lower levels (Table 6.1).

Two and 6 months after amendment application, we observed a noticeable decrease in Pb and Zn bioavailability, as compared to control plots (Fig. 6.1). The reduction in metal mobility was more efficient in SITE 2 and 3, interestingly the sites initially showing a higher toxicity. This reduction could be due to the presence of components from amendments (Ross, 1994) more than a vegetation effect, since this response was observed in both vegetated and initially non-vegetated soils. The low metal bioavailability observed in SITE 4 can be related with its higher soil pH value (Wong et al., 2003; Galende et al., *in preparation*, Chapter 1 and 2). Among amendments, PAPER was the amendment which most contributed to the reduction of metal bioavailability in initially non-vegetated sites, whilst in vegetated sites, COW-treated plots showed the highest decrease in metal bioavailability (Fig. 6.1).

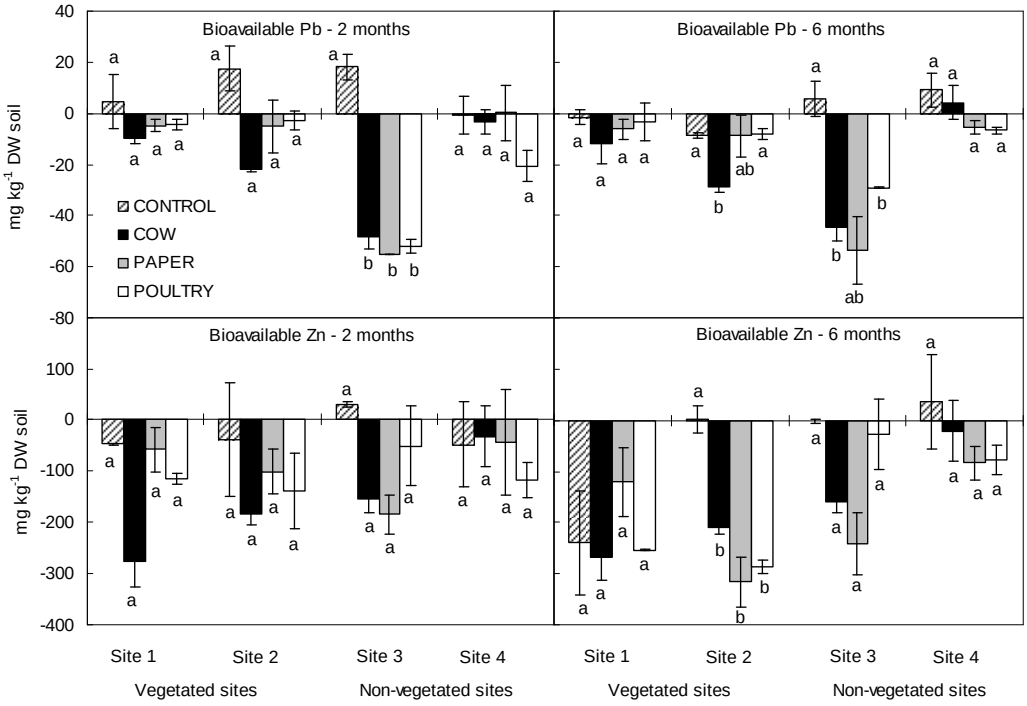

Fig. 6.1. Differences of soil metal bioavailability between the beginning of the study (0 months) and 2 and 6 months later. PAPER: paper mill sludge mixed with poultry manure; POULTRY: poultry manure treated soils; COW: cow slurry.

6.4.3. Effect of amendments on vegetation

Festuca rubra plants have been proposed as suitable species for phytostabilization. After all, *Festuca rubra* is a metal excluder, with an extensive root system, a large amount of biomass and a low root-to-shoot metal translocation (Epelde et al., 2010). As a dominant native plant in the studied mine, *F. rubra* tolerates a shortage of nutrients and the presence of high levels of metals. All amendments applied in vegetated sites (SITES 1, 2) greatly improved *F. rubra* vegetation, as indicated by the growth index (Table 6.3), especially in SITE 2. Two months after amendment application, POULTRY had a better

performance, but this effect progressively decreased, probably due to the presence of a form of N more readily available for plants (data not shown), as observed on a microcosm study of phytostabilization with this amendment (Galende et al., *in preparation*, Chapter 2). Finally, great differences among amendments were not found regarding plant growth, indicating that plants responded mainly to the N supply of the amendments, as we applied different amounts of amendments in order to finally reach the same amount of N. Besides plant performance, one important aspect in phytostabilization processes is the reduction in metal phytoavailability. All amendments reduced metal (Pb, Zn) concentrations in shoots of *F. rubra* (Table 6.4). The most effective amendments were PAPER and COW for SITES 1 and 2, respectively. This reduction was a consequence of both (i) reduction in metal bioavailability (Fig. 6.1) and (ii) promotion of plant growth, provoking a dilution of metal concentration by growth (Shen et al., 1997).

Table 6.3. Growth index (calculated on a dry weight basis) of *Festuca rubra* biomass in vegetated sites and vegetation cover in initially non-vegetated areas at the end of the study (6 months).

	Vegetated areas Growth index		**Non-vegetated areas Vegetation cover (%)**	
	Site 1	**Site 2**	**Site 3**	**Site 4**
CONTROL	1.4	1.7	2.2	6.8
COW	2.4	3.2	12	11.0
PAPER	1.7	3.6	12.4	14.0
POULTRY	2.6	4.3	23.7	19.0

PAPER: paper mill sludge mixed with poultry manure
POULTRY: poultry manure treated soils
COW: cow slurry

In non-vegetated soils, after transplanting of *F. rubra* explants and amendment application, plant growth and spontaneous colonization of new species was stimulated (Table 6.3). Vegetation cover of control soils only reached 2.2% and 6.8% in SITES 3 and 4, respectively. Interestingly, POULTRY-treated

plots showed the highest values of vegetation cover: 23.7 and 19% of plot area in SITES 3 and 4, respectively. As indicated for plant growth, N form and bioavailability in POULTRY-treated plots could be also responsible for stimulating plant colonization of non-vegetated soils. This aspect needs more attention in future studies. Among plots, *F. rubra* and *Pteridium aquilinum* spontaneously grew after amendment application in SITES 3 and 4, *Agrostis capillaris* in SITE 3, and *Plantago lanceolata*, *Rumex acetosa* and *Silene vulgaris* in SITE 4. All these species belonged to the plant biodiversity of the area (Barrutia et al., 2011). Moreover, the most beneficial effects were obtained in SITE 3, which originally presented a higher toxicity for biological indicators. Besides amendments, the appropriate weather conditions during the study (mean temperature of 14.2 ºC and total rainfall of 296 l m^{-2} (Euskalmet, 2010) could also contribute to plant growth and colonization of non-vegetated soils. Moreover, initial soil tillage could also improve plant growth in treated and control plots, by reducing compaction and bulk density and increasing porosity, which promoted seed germination and seedling development, as previously described by Kabas et al. (2012).

Table 6.4. Lead and zinc concentrations in shoots and accumulative phytoextraction of *Festuca rubra* at the end of the experiment. Statistical analyses were performed for each parameter and treatment. Different letters indicate statistically significant differences among means ($p<0.05$).

	Site 1				Site 2			
	Metal in shoots (mg kg^{-1} DW)		Phytoextraction (mg m^{-2})		Metal in shoots (mg kg^{-1} DW)		Phytoextraction (mg m^{-2})	
	Pb	Zn	Pb	Zn	Pb	Zn	Pb	Zn
CONTROL	40.8 ± 5.5 a	417 ± 57 a	0.41 ± 0.06 a	4.17 ± 0.57 a	62.7 ± 10.4 a	590 ± 175 ab	4.71 ± 0.38 a	36.01 ± 7.80 a
COW	21.3 ± 7.4 a	249 ± 29 b	1.76 ± 0.50 a	19.74 ± 2.73 b	49.0 ± 5.9 a	287 ± 11 a	7.06 ± 0.77 a	47.13 ± 3.94 a
PAPER	19.5 ± 2.8 a	149 ± 13 b	1.42 ± 0.25 a	12.03 ± 1.78 b	44.8 ± 10.9 a	483 ± 20 b	8.91 ± 1.57 a	79.17 ± 6.17 b
POULTRY	42.7 ± 9.4 a	279 ± 38 b	6.03 ± 1.69 b	44.2 ± 7.68 c	40.4 ± 7.1 a	407 ± 104 ab	8.51 ± 2.04 a	67.37 ± 18.03 ab

PAPER: paper mill sludge mixed with poultry manure

POULTRY: poultry manure treated soils

COW: cow slurry

In any case, growth stimulation of established vegetation and spontaneous colonization by plants contribute to an increase in root exudates, which can be consumed by soil microbial communities, favoring soil biological health and remediation (Tejada et al., 2006). Therefore, the development of a new rhizosphere environment could have an important role in metal speciation (Mastretta et al., 2006), contributing to decrease metal bioavailability (Fig. 6.1) and phytoavailability (Table 6.4), and to reduce plant toxicity (Table 6.3).

6.4.4. Effect of amendments on biological indicators of soil health

The above results indicate that organic amendments reduced metal bioavailability, phytoavailability and improved plant performance. However, the ultimate goal of any phytoremediation process must be not only to reduce the contaminant from the soil but, most importantly, to recover soil health, i.e. the capacity of the soil to sustainably carry out its functions (Doran and Safley, 1997). Soil biological properties, particularly those related to the biomass, activity and diversity of soil microbial communities, and ecotoxicity plant bioassays have a great potential as bioindicators of soil health. Biological indicators complement soil chemical characterization in order to assess potential ecological risks in contaminated soils (Alvarenga et al., 2008b).

Several studies have described that toxic metal concentrations negatively affect microbial communities by lowering their activity, biomass, functional stability and diversity (see review by Gómez-Sagasti et al., 2012). On the other hand, when a strong pressure is exerted on microorganisms exposed to toxic metals for long periods of time (as in mine soils), this results in the selection of the most tolerant microorganisms by the development of intracellular and extracellular resistance mechanisms (Bruins et al., 2000). In the present study, we observed an increase in soil microbial parameters in both vegetated and initially non-vegetated soils treated with amendments (Tables 6.5 and 6.6). The

beneficial effects were due to OM and nutrients input (as observed by Tejada et al., 2006) and decrease in metal bioavailability. Potentially active microbial biomass (measured as SIR) was more stimulated by amendments than overall microbial activity (measured as BR). As pointed out for vegetation, the benefits were higher in those sites initially most affected (SITES 2 and 3). In vegetated and initially non-vegetated sites, COW treatment was best at maintaining higher values of microbial parameters, as compared to initial values and non-amended soils. In general, increases in microbial parameters were more noticeable in initially non-vegetated sites (Tables 6.5 and 6.6). However, although organic amendments increased soil microbial properties in initially non-vegetated soil, those values are far below those observed in non-amended vegetated soils (Tables 6.5 and 6.6). In consequence, vegetation appears to be the main factor regarding the stimulation of microbial communities in our mine soil.

The beneficial effects of all amendments were also observed during the root elongation bioassay with *L. sativa* (Tables 6.5 and 6.6). As indicated for bioavailable metals in soil (Fig. 6.1), the most effective amendment in terms of the stimulation of root elongation was PAPER in initially non-vegetated soils and COW in vegetated ones (Tables 6.5 and 6.6). In fact, we found linear negative regressions between *L. sativa* root elongation and metal bioavailability ($p<0.01$, $R2 = 0.585$ and 0.702 for Pb and Zn, respectively, in vegetated sites and 0.549 and 0.727 for Pb and Zn, respectively, in initially non-vegetated sites). This result, together with other studies (Galende et al., *in preparation*, Chapters 1 and 2), confirms the usefulness of the elongation bioassay for the assessment of metal bioavailability in phytostabilization processes under field conditions.

Table 6.5. Soil biological parameters in vegetated sites at the beginning and at the end of the experiment. Statistical analyses were performed for each parameter, sampling time and treatment. Different letters indicate statistically significant differences among means ($p<0.05$).

	SITE 1		SITE 2	
	0 months	6 months	0 months	6 months
Basal Respiration (mg C kg^{-1} DW soil h^{-1})				
CONTROL	9.0 ± 0.1 a	8.3 ± 0.4 a	5.8 ± 0.1 a	5.9 ± 0.1 a
COW	8.3 ± 0.2 a	9.0 ± 1.5 a	5.2 ± 0.0 a	6.5 ± 0.8 a
PAPER	9.1 ± 0.0 a	8.9 ± 0.2 a	5.1 ± 0.2 a	5.8 ± 0.4 a
POULTRY	8.7 ± 0.2 a	8.7 ± 0.8 a	4.4 ± 0.1 a	4.9 ± 0.1 b
SIR (mg C kg^{-1} DW soil h^{-1})				
CONTROL	47.8 ± 1.3 a	61.4 ± 0.2 a	17.5 ± 0.4 a	22.7 ± 1.5 b
COW	35.0 ± 1.2 a	69.4 ± 7.7 b	16.8 ± 0.7 a	25.6 ± 1.1 b
PAPER	47.4 ± 1.4 a	63.9 ± 2.4 b	17.9 ± 1.3 a	20.0 ± 2.2 a
POULTRY	48.7 ± 1.8 a	66.7 ± 2.2 b	19.7 ± 1.0 a	24.7 ± 1.4 a
Arylsulphatase (mg NP kg^{-1} DW soil h^{-1})				
CONTROL	448.7 ± 0.4 a	464.9 ± 11.1 a	255.3 ± 2.4 a	338.8 ± 17.7 b
COW	413.9 ± 4.5 a	371.7 ± 65.1 a	242.7 ± 10.7 a	300.0 ± 34.3 a
PAPER	791.8 ± 4.0 a	506.5 ± 28.9 a	179.7 ± 3.8 a	237.7 ± 12.2 b
POULTRY	400.3 ± 7.9 a	461.6 ± 13.8 b	281.4 ± 8.8 a	263.7 ± 4.3 a
Acid phosphatase (mg NP kg^{-1} DW soil h^{-1})				
CONTROL	875.4 ± 4.7 a	837.6 ± 51.2 a	549.7 ± 14.2 a	573.6 ± 32.5 a
COW	972.2 ± 52.9 a	901.1 ± 234.7 a	388.7 ± 21.6 a	357 ± 12.7 b
PAPER	972.7 ± 10.8 a	851.6 ± 23.0 b	397.9 ± 18.2 a	347.8 ± 17.3 a
POULTRY	1163.8 ± 5.3 a	946.7 ± 120.7 a	456.5 ± 9.1 a	426.7 ± 23.5 a
β-Glucosidase (mg NP kg^{-1} DW soil h^{-1})				
CONTROL	497.6 ± 12.2 a	652.6 ± 16.0 b	222.7 ± 2.3 a	361.1 ± 16.2 b
COW	574.9 ± 0.4 a	595.7 ± 73.5 a	209.3 ± 0.6 a	321.7 ± 11.0 b
PAPER	474.4 ± 0.7 a	572.3 ± 22.2 b	192.3 ± 0.6 a	217.8 ± 10.2 a
POULTRY	435.2 ± 8.3 a	581.6 ± 18.4 b	232.9 ± 4.3 a	263.8 ± 9.7 a
Urease (mg N-$NH4^{+}$ kg^{-1} DW soil $2h^{-1}$)				
CONTROL	71.6 ± 3.5 a	101.7 ± 2.6 b	20.2 ± 0.5 a	38.5 ± 1.3 b
COW	64 ± 1.3 a	101.4 ± 18.3 a	26.5 ± 0.5 a	44.7 ± 5.2 b
PAPER	61.9 ± 1.3 a	110.4 ± 1.4 b	19.7 ± 0.6 a	32.5 ± 2.0 b
POULTRY	65.8 ± 1.0 a	95.3 ± 3.2 b	26.7 ± 0.1 a	33.2 ± 2.2 a
FDA hydrolysis (mg NaF kg^{-1} DW soil h^{-1})				
CONTROL	297.4 ± 0.6 a	272.6 ± 6.7 b	171.3 ± 12.9 a	191.6 ± 7.8 a
COW	304.4 ± 4.9 a	256.4 ± 7.0 b	144.8 ± 3.4 a	171.6 ± 13.1 a
PAPER	294.4 ± 0.5 a	248.0 ± 5.3 b	112.4 ± 4.3 a	168.8 ± 11.2 b
POULTRY	296.5 ± 2.9 a	277.4 ± 9.5 a	136.1 ± 4.4 a	150.0 ± 3.4 a
OEA				
CONTROL	334 ± 4 a	371 ± 6 b	161 ± 1 a	220 ± 4 b
COW	339 ± 5 a	344 ± 51 a	150 ± 2 a	192 ± 14
PAPER	334 ± 3 a	368 ± 10 b	125 ± 0 a	158 ± 7 b
POULTRY	331 ± 5 a	366 ± 7 b	161 ± 2 a	171 ± 3 a
Root elongation (mm)				
CONTROL	13.0 ± 0.8 a	17.3 ± 0.5 b	8.7 ± 0.3 a	9.2 ± 0.2 a
COW	14.1 ± 0.7 a	21.8 ± 0.5 b	7.7 ± 0.2 a	10.8 ± 0.3 b
PAPER	14.3 ± 0.5 a	20.7 ± 30.8 b	8.9 ± 0.4 a	10.5 ± 0.3 b
POULTRY	10.9 ± 0.4 a	15.7 ± 0.5 b	10.3 ± 0.5 a	9.3 ± 0.2 a

FDA: fluorescein diacetate; NaF: fluorescein sodium salt; NP: 4-nitrophenol; OEA: overall enzyme activity; SIR: substrate-induced respiration; PAPER: paper mill sludge mixed with poultry manure; POULTRY: poultry manure treated soils; COW: cow slurry.

Table 6.6. Soil biological parameters in initially non-vegetated sites at the beginning and at the end of the experiment. Statistical analyses were performed for each parameter, sampling time and treatment. Different letters indicate statistically significant differences among means ($p<0.05$).

	SITE 3		SITE 4	
	0 months	6 months	0 months	6 months
Basal Respiration (mg C kg^{-1} DW soil h^{-1})				
CONTROL	1.1 ± 0.0 a	0.9 ± 0.1 a	3.1 ± 0.1 a	2.3 ± 0.2 b
COW	0.9 ± 0.1 a	1.1 ± 0.3 a	3.7 ± 0.0 a	3.6 ± 0. 2 a
PAPER	1.4 ± 0.1 a	1.5 ± 0.1 a	3.4 ± 0.0 a	2.7 ± 0.2 b
POULTRY	2.4 ± 0.1 a	0.8 ± 0.1 b	2.7 ± 0.2 a	2.1 ± 0.0 a
SIR (mg C kg^{-1} DW soil h^{-1})				
CONTROL	3.9 ± 1.3 a	7.7 ± 0.4 a	4.0 ± 0.2 a	6.0 ± 0.8 a
COW	2.6 ± 0.6 a	6.6 ± 1.6 a	4.4 ± 0.4 a	9.4 ± 2. 2 a
PAPER	5.6 ± 1.7 a	8.1 ± 0.8 a	5.0 ± 0.7 a	6.5 ± 0.6 a
POULTRY	5.3 ± 0.4 a	7.1 ± 0.1 a	3.1 ± 0.1 a	6.2 ± 0.9 a
Arylsulphatase (mg NP kg^{-1} DW soil h^{-1})				
CONTROL	10.7 ± 0.2 a	12.7 ± 0.1 b	21.8 ± 0.1 a	16.6 ± 3.1 a
COW	11.3 ± 0.2 a	12.2 ± 1.5 a	35.4 ± 0.1 a	19.4 ± 4.0 b
PAPER	7.1 ± 0.3 a	8.5 ± 0.8 a	21.2 ± 0.9 a	18.4 ± 2. 1 a
POULTRY	5.9 ± 0.1 a	7.7 ± 0.2 b	7.4 ± 0.1 a	8.6 ± 0.4 a
Acid phosphatase (mg NP kg^{-1} DW soil h^{-1})				
CONTROL	172.5 ± 10.8 a	142.5 ± 6.7 a	160.6 ± 5.7 a	131.0 ± 7.9 a
COW	107.4 ± 6.9 a	93.8 ± 2.0 a	179.6 ± 5.0 a	197.3 ± 14.6 a
PAPER	116.8 ± 2.2 a	100.7 ± 3.0 b	159.8 ± 4.6 a	122.3 ± 1.1 a
POULTRY	100.6 ± 4.3 a	84.8 ± 3.3 b	138.9 ± 2.0 a	140.4 ± 3.2 a
β-Glucosidase (mg NP kg^{-1} DW soil h^{-1})				
CONTROL	30.8 ± 0.4 a	49.3 ± 1.9 b	56.4 ± 0.5 a	66.5 ± 5.9 a
COW	15.1 ± 0.5 a	51.8 ± 6.3 b	67.6 ± 0.6 a	131.4 ± 31.5 a
PAPER	26.8 ± 1.9 a	43.1 ± 7.9 a	75.7 ± 3.6 a	72.4 ± 13.4 a
POULTRY	14.3 ± 0.0 a	41.0 ± 1.9 b	87.8 ± 1.9 a	100.8 ± 19.1 a
Urease (mg N-$NH4^{+}$ kg^{-1} DW soil $2h^{-1}$)				
CONTROL	2.2 ± 0.1 a	3.3 ± 0.6 a	3.5 ± 0.4 a	5.8 ± 1.1 a
COW	2.1 ± 0.4 a	4.6 ± 0.4 b	6.7 ± 0.2 a	11.3 ± 1.8 a
PAPER	0.6 ± 0.3 a	5.7 ± 0.1 b	7.2 ± 0.1 a	6.2 ± 0.6 a
POULTRY	2.5 ± 0.1 a	4 ± 0.4 a	6.8 ± 0.1 a	8.2 ± 1.6 a
FDA hydrolysis (mg NaF kg^{-1} DW soil h^{-1})				
CONTROL	<dl a	26.8 ± 1.3 b	52.0 ± 0.4 a	34.7 ± 2.0 b
COW	<dl a	27.1 ± 2.1 b	25.9 ± 5.4 a	37.0 ± 9.5 a
PAPER	<dl a	27.4 ± 2.4 b	<dl a	32.6 ± 1.8 a
POULTRY	<dl a	<dl a	<dl a	30.6 ± 4.6 b
OEA				
CONTROL	17 ± 0 a	24 ± 9 b	32 ± 0 a	30 ± 1 a
COW	13 ± 1 a	24 ± 2 b	37 ± 2 a	46 ± 9 a
PAPER	10 ± 1 a	22 ± 1 b	31 ± 2 a	31 ± 2 a
POULTRY	12 ± 0 a	16 ±1 b	23 ± 0 a	31 ± 4 a
Root elongation (mm)				
CONTROL	6.2 ± 0.2 a	8.2 ± 0.2 b	12.1 ± 0.2 a	11.5 ± 0.4 b
COW	6.8 ± 0.2 a	13.0 ± 0.4 b	12.2 ± 0.3 a	17.3 ± 0.5 b
PAPER	7.0 ± 0.3 a	21.6 ± 1.5 b	13.5 ± 0.3 a	23.2 ± 1.0 b
POULTRY	8.2 ± 0.3 a	9.2 ± 0.3 a	8.0 ± 0.4 a	11.7 ± 0.4 b

dl: detection limit;. FDA: fluorescein diacetate; NaF: fluorescein sodium salt; NP: 4-nitrophenol; OEA: overall enzyme activity; SIR: substrate-induced respiration; PAPER: paper mill sludge mixed with poultry manure; POULTRY: poultry manure treated soils; COW: cow slurry.

6.4.5. Mine soil heterogeneity

In the PCA (Fig. 6.2), vegetated and initially non-vegetated sites were clearly separated, reflecting their heterogeneity, as frequently observed in mine tailings (Cobb et al., 2000; Córdova et al., 2011; Ernst, 2005). This explains the

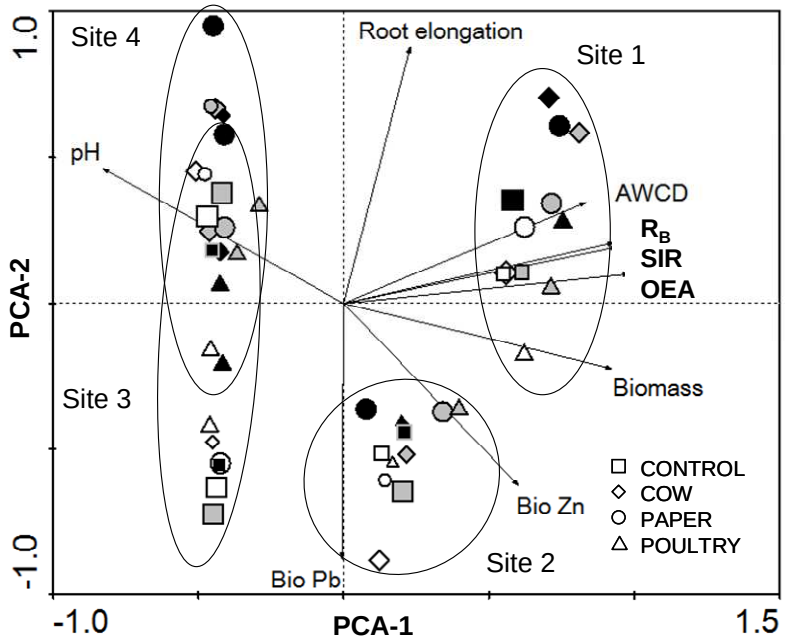

Fig. 6.2. Biplot of PCA performed on soil chemical parameters (pH and bioavailable Pb and Zn), soil microbial parameters (AWCD, R_B, SIR and OEA), bioassays with *L. sativa* (root elongation) and plant biomass. Each point represents the mean value of samples from vegetated (site 1 and 2) and initially non-vegetated sites (site 3 and 4) before amendments application and two and six months later. PCA-1 accounts for 60% of the variance, while PCA-2 accounts for 27% of the variance. AWCD: average well color development; R_B: basal respiration; SIR: glucose-induced respiration; OEA: overall enzyme activity; bio: bioavailable. White color: initial sampling; grey color: spring sampling; black color: summer sampling. PAPER: paper mill sludge mixed with poultry manure; POULTRY: poultry manure treated soils; COW: cow slurry.

different response of each site to the same treatment and represents one of the main problems for the rehabilitation of the mine tailings. In the PCA, vegetated areas with a higher plant biomass appear closer to soil microbial properties. This can due to the higher content of OM: the presence of root exudates from vegetation will be used as substrates by soil microbial communities and microbial colonization of plant roots (Lynch and Whipps, 1990; Delorme et al., 2001). Non-vegetated soils were characterized by harsh conditions and presented lower values of biological parameters than vegetated soil. Similarly, Epelde et al. (2008a) reported lower rates of microbial biomass and activity in bare mine soils when compared with vegetated mine soils. In these soils, edaphic factors such as an elevated pH could greatly reduce metal bioavailability. All the amendments improved soil physiochemical and biological parameters, and hence soil health. However, as indicated by PC-2, the amendment-induced beneficial effects were more noticeable in initially non-vegetated soils.

6.5. Conclusions

Amendment application increased soil OM and nutrient content, especially in initially non-vegetated soils, decreased metal bioavailability, and facilitated plant colonization and the growth of native plants. The most successful amendments at decreasing metal bioavailability were COW in vegetated areas and PAPER in initially non-vegetated areas. These treatments resulted in lower Pb and Zn concentrations in *F. rubra* leaves from vegetated sites. POULTRY was the best treatment for plant growth and colonization in initially non-vegetated sites. Taking into account soil microbial parameters, COW was the best treatment in both vegetated and initially non-vegetated sites. Improvements in plant growth and soil microorganisms were higher in initially most affected soils. The root elongation bioassay with *Lactuca sativa* proved to be a good bioindicator of soil metal bioavailability in phytostabilization processes under field conditions.

7. Chemophytostabilization: a case study from northern Spain

Más yo quiero mirarte, primavera,
verde y florida solo, toda verde
como si el mundo, si la tierra toda
despertase un jardín, un inicial vergel
de altas espigas llenas y frutales.

(Rafael Alberti)

7. Chemophytostabilization: a case study from northern Spain

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7.1. Abstract

Chemophytostabilization (i.e., the use of plants and organic or inorganic amendments to reduce metal mobility and bioavailability) is an eco-friendly phytotechnology with great potential for the remediation of metal-polluted mine soils. However, this remediation strategy is still limited by a lack of knowledge on the interactions among the soil matrix, metal pollutants, amendments, plants and rhizosphere microbial communities. In order to assess interactions between those factors, we carried out a chemophytostabilization study in a Pb-Zn abandoned mine from northern Spain to evaluate the effect of two organic amendments (cow slurry, poultry manure) and the excluder plant *Festuca rubra* L. on rhizosphere microbial communities, under microcosm (pot) and field conditions. While the amendments enhanced the rhizosphere microbial properties measured in the microcosm experiment, this effect was less evident in the field experiment due to the high heterogeneity of the studied mine soil. It was thus concluded that results from pot studies are not easily extrapolated to field conditions and that long-term follow-up monitoring programs are needed to properly assess the effectiveness of chemophytostabilization processes.

7.2. Introduction

Soils provide essential ecosystem services and goods. Unfortunately, during the last century, mining activities have contributed to soil metal pollution, a well-known worldwide environmental problem. As a result, lead-zinc mine tailings frequently have a poor physical structure, high levels of total and bioavailable metals and low nutrient concentrations (Ye et al., 2002). Chemophytostabilization or aided phytostabilization (i.e., the use of plants and their associated rhizosphere microorganisms, together with the application of organic or inorganic amendments, to reduce metal mobility and bioavailability) is an environmentally friendly phytotechnology with great potential for the remediation of metal-polluted mine soils. However, this remediation strategy is still limited by a lack of knowledge on the interactions among the soil matrix, metal pollutants, amendments, plants and rhizosphere microbial communities. In any case, the aim of a chemophytostabilization process must be not only to reduce metal mobility and bioavailability, but also to restore soil health.

Historically, mining has been one of the most important industrial activities in the Basque Country (northern Spain). In fact, for centuries, the industrial significance of mining exploitation in this region (mostly, of iron, but also of zinc, lead, etc.) has resulted in a profound transformation of its natural, social and economical scenery. As a consequence, nowadays, abandoned mining areas can be found throughout the Basque landscape, particularly in the province of Biscay.

Within the context of the chemophytostabilization of a Pb/Zn-contaminated mine soil from Biscay, the aim of this study was to study the effects of metallicolous population of *Festuca rubra* L. and two organic amendments (cow slurry and poultry manure) on functional diversity of microbial communities under controlled (microcosm) and natural (field) conditions.

7.3. Materials and Methods

7.3.1. Experimental design

In the microcosm study, two organic amendments from livestock (cow slurry and poultry manure) were mixed with a mine soil with high levels of Pb and Zn. Five weeks after amend, plants of *Festuca rubra*, native from the mining area, were planted and allowed to growth under controlled greenhouse conditions for another 18 weeks. The effect of chemophytostabilization treatments on rhizosphere microbial communities was studied. Bacterial and fungal gene abundances were monitored by quantitative real-time PCR following Lueders et al. (2004a,b) and Yergeau et al. (2007), and community-level physiological profiles (CLPP) were obtained with Biolog EcoPlates^{TM,} according to Epelde et al. (2008).

We also performed an *in-situ* chemophytostabilization field experiment in the "Coto Txomin" mine using the same two amendments (poultry manure and cow slurry) for CLPP analysis. In this case, amendments were superficially applied to three sites of the mining area, vegetated by *F. rubra*, and which exhibited different levels of bioavailable Pb and Zn. Samples were taken at three different times: just before the application of amendments and two and six months later.

7.3.2. Site description

The "Coto Txomin" mine, as it is locally known, is an abandoned Pb-Zn mining area located in the Western Biscay district, Basque-Cantabrian Basin, northern Spain (43º 13´ N; 3º 26´ W) with a temperate Atlantic climate. The hypogene assemblage is composed of sphalerite, galena, pyrite, dolomite, calcite and quartz (Grandia et al., 2003). Lead and zinc extraction by open cast mining ceased in the late 1970s. At the present time, the mining area includes open pits, piles of waste rock and overburden surfaces, tailing dams and degraded zones affected by mining subsidence (for a more detailed site description and

characterization, see Barrutia et al., 2011). Despite the harsh conditions of the mine soil in that area, over the last 30 years, the studied mining area has been naturally revegetated with a variety of plant species that show different physiological strategies to deal with soil metal pollution (Barrutia et al., 2011): hyperaccumulators such as *Noccaea caerulescens* J. Presl. C. Presl. (formerly, *Thlaspi caerulescens* J. Presl. C. Presl.), accumulators and indicators such as *Jasione montana* L. and *Rumex acetosa* L., excluders such as *Festuca rubra* L. and *Ulex europaeus* L., etc.

In the "Coto Txomin" mine soil, extremely high values of total metal concentrations have been detected, up to 84, 58 928 and 203 110 mg kg^{-1} DW soil of Cd, Pb and Zn, respectively (Barrutia et al., 2011; Epelde et al., 2010). The soil is characterized as sandy loam and organic matter concentrations range between 0.8 and 10.8%, and pH ranges from 5.6 to 7.1. This soil is also rather deficient of essential plant nutrients (Barrutia et al., 2011), as previously reported for other mining areas (Bradshaw, 1997; Li, 2006; Tordoff et al., 2000; Wong, 2003).

7.3.3. Statistical analysis

Every group of data was checked for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Cochran C test) and, when possible, differences between treatment means were analyzed by one-way ANOVA followed by Duncan test ($p<0.05$). Differences among data not satisfying homogeneity of variance were analysed using Student's t test ($p<0.05$). A redundancy analysis (RDA) was performed to evaluate the relationships between metal bioavailability and substrate consumption in the Biolog EcoPlate™. All statistical analyses were carried out with the SPSS v. 15.0 software, except for RDA (Canoco v. 4.5 sotfware).

7.4. Results and Discussion

In recent years, we have carried out several microcosm and field experiments with soil from the abovementioned mining area to investigate the interactions between plants and rhizosphere microbial communities, within the context of the phytoremediation of metal-polluted mine soils. We examined the effectiveness of phytostabilization with *Lolium perenne* plus the addition of a synthetic (Calcinit + urea + PK 14% + calcium carbonate) or organic (cow slurry) amendment (Epelde et al., 2009). In a short-term pot study, we observed an increase in the biomass (microbial biomass C), activity (enzyme activities, potentially mineralizable N) and functional diversity (Biolog Ecoplates^{TM} parameters) of rhizosphere microbial communities in response to these treatments. The beneficial effects on rhizosphere microbial properties, and hence soil health, were more accentuated in organically-amended than in synthetically-amended soils.

In this work, we first studied the ability of *Festuca rubra* and two organic amendments (cow slurry, poultry manure) for chemophytostabilization of the "Coto Txomin" mine soil. At the end of the experiment, the abundance of bacterial 16S rRNA and fungal 18S rRNA gene copies (Fig. 7.1A) was generally higher in amended as compared to untreated soil. Likewise, although differences were not statistically significant, the addition of both poultry manure and cow slurry appeared to increase fungi:bacteria ratios (Fig. 7.1B). This phenomenon could be due to a higher content of aromatic compounds in amended soils at the end of the experiment from the decomposition of organic matter, as these recalcitrant compounds are preferably degraded by fungal populations (Grayston et al., 2004). Although the relative abundance of bacteria and fungi in ecosystems has received a large amount of attention, little is yet known about the functional significance of this ratio (van der Heijden et al., 2008). In general terms, bacteria-dominated food webs might enhance rates of nutrient mineralization and availability of nutrients to plants, whereas fungal-dominated food webs promote slow and highly conservative cycling of nutrients (Wardle et al., 2004).

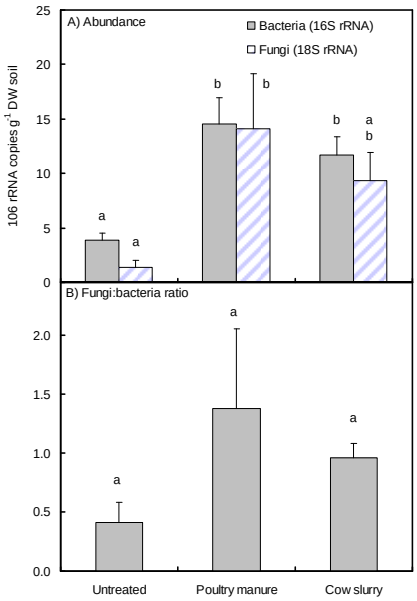

Fig. 7.1. Effect of chemophytostabilization treatments (untreated, poultry manure and cow slurry) on (A) gene abundance of bacteria and fungi and (B) fungi:bacteria ratio, at the end of the experiment (n = 4, mean ± SE). Different letters indicate significant ($p<0.05$) differences among treatments.

Community-level physiological profiles (CLPPs), just after the addition of the amendments, generally showed higher rates of carbon substrate utilization in amended as compared to untreated soils. This was also the case for poultry manure-amended *versus* cow slurry-amended soils (Fig. 7.2A). The short-term stimulation of the heterotrophic bacterial populations derived from the application of organic amendments was probably responsible for the higher values found in amended *versus* untreated soils (Epelde et al., 2009). In addition, the amount of nitrogen was 2.4-fold higher in the poultry manure than in the cow slurry (see Chapter 5), which might at least partially explain the higher values of carbon substrate utilization in poultry manure-amended soil. On the other hand, the capacity of rhizosphere microbial communities to use carbon substrates showed a marked reduction over time in both amended and untreated soils (Figs. 7.2B

and C). This observed decrease could be due to our specific controlled experimental conditions, which appeared to reduce the capacity of the soil microbial community to use different carbon sources (i.e., its functional diversity).

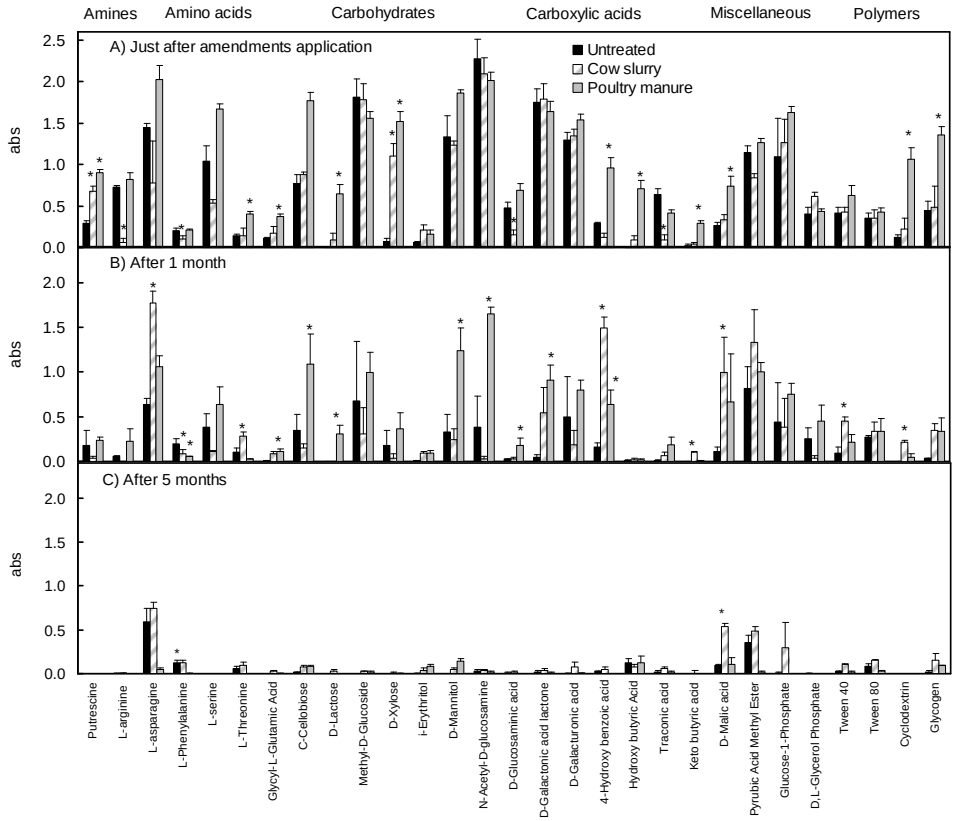

Fig. 7.2. Metabolic fingerprints of carbon substrate utilization patterns obtained with Biolog Ecoplates^{TM} at 40 h incubation time ***(n = 3, mean ± SE)***. (A) Just after amendment application, (B) At the end of the 1-month stabilization period, and (C) At the end of the 4-month chemophytostabilization experiment. Only those substrates showing significant ($p<0.05$) differences among treatments or over time are presented. Asterisks indicate significant ***(p<0.05)*** differences between amended soils and corresponding untreated mine soil***.***

In this regard, we performed the *in-situ* chemophytostabilization field experiment in the "Coto Txomin" mine using the same two amendments (poultry manure and cow slurry) in three sites. SITE 1 had 47 and 827 mg kg^{-1} DW soil of

bioavailable Pb and Zn, respectively; SITE 2 had 41 and 906 mg kg^{-1} DW soil of bioavailable Pb and Zn, respectively; and SITE 3 had 63 and 1 224 mg kg^{-1} DW soil of bioavailable Pb and Zn, respectively.

A redundancy analysis was performed (Fig. 7.3) with data on bioavailable Pb and Zn concentrations and CLPPs obtained with Biolog EcoPlates™ from the three vegetated areas for the three different sampling times. According to the RDA analysis, bioavailable metal (Pb, Zn) concentrations explained 24% of the variability observed in Biolog EcoPlates™ data. Bioavailable Zn concentration most negatively affected CLPPs data, possibly due to its higher bioavailability. The impact of metals was lowest for SITE 1, which showed higher values of carbon substrate utilization than the other two sites.

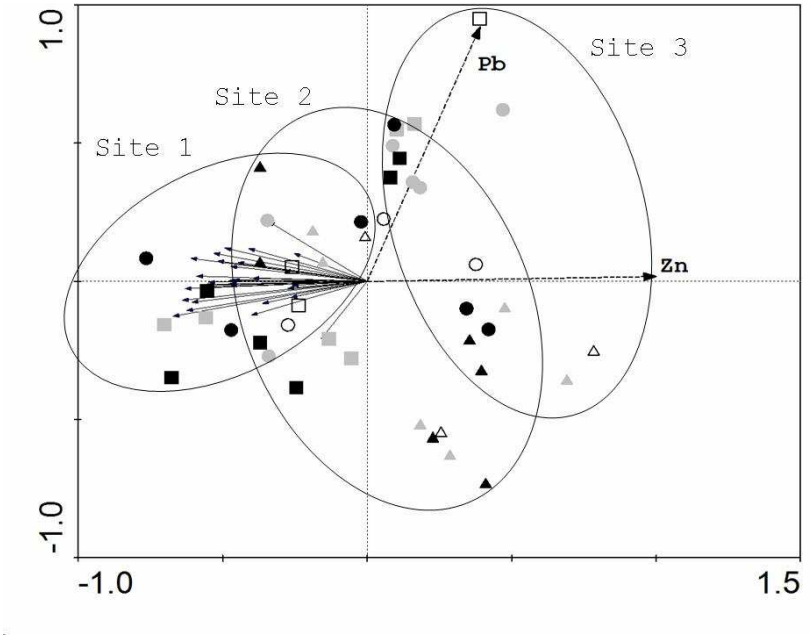

Fig. 7.3. Triplot of the RDA performed on carbon substrate utilization data as response variable (solid vectors) and soil Pb and Zn bioavailable concentrations as explanatory variables (dashed vectors). Circle: untreated mine soils; Square: Cow slurry-treated soils; Triangle: Poultry manure-treated soils; Open symbol: before amendment application; Closed grey symbol: two months after amendment application; Solid black symbol: six months after amendment application.

Indeed, sample distribution in the triplot depended more on sampling site than on sampling time or type of amendment, reflecting the high heterogeneity of the studied mine. In this sense, the heterogeneity of metalliferous mining sites is an important issue to be taken into account in long-term chemophytostabilization monitoring programs and also to explain uncertainties related with metal toxicity and ecological impact.

7.3. Conclusions

Amendments enhanced CLPP of rhizosphere microbial properties measured in the microcosm experiment, while this effect was less clear in the field experiment due to the high heterogeneity of the studied mine soil. This confirms that results from pot studies are not easily extrapolated to field conditions and that the establishment of follow-up monitoring programs is essential to ensure the long-term effectiveness of chemophytostabilization programs in terms of both reduction of metal bioavailability and the recovery of soil health.

Chemophytostabilization using amendments and excluder plants is a promising phytotechnology for the remediation of metal polluted mine soils, particularly regarding soil health recovery. However, before opting for chemophytostabilization as the remediation strategy of choice, pot and small-scale *in-situ* field experiments should be carried out in order to properly select suitable amendments, plant species and their corresponding optimal growth conditions for the specific metal-polluted mine area. A thorough examination of plant-rhizosphere microorganism interactions is key to establishing the necessary mechanistic understanding to predict remediation success and monitor the restoration of biological soil health parameters.

8. Antioxidant and photoprotective responses induced by Pb and Cd in Lemna minor L. is not caused by δ-aminolevulinic acid accumulation

8. Antioxidant and photoprotective responses induced by Pb and Cd in *Lemna minor* L. is not caused by δ-aminolevulinic acid accumulation

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8.1. Abstract

Heavy metals cause oxidative stress in plants and activate antioxidant mechanisms. It has been suggested that Cd inhibit δ-aminolevulinic acid dehydratase (ALAD) in plants, generating an accumulation of δ-aminolevulinic acid (δ-ALA), an intermediary of porphyrins biosynthetic pathway, which would be responsible for the oxidative stress. This effect has also been described for Pb in animals, but it has not been studied in plants yet. The aim of this study is to evaluate the effects of δ-ALA accumulation in plant tissues caused by Pb and Cd and its relationship with the antioxidant and photoprotective systems of *Lemna minor* L. For this purpose, *L. minor* colonies were exposed to 50 µM Pb, 5 µM Cd, 0.1 mM 4,6-dioxoheptanoic acid (DA) or 0.1 mM exogenous δ-ALA (ALA) for 4 days. A decrease of 50-59% of growth was observed in all treatments, except for ALA. Chlorosis symptoms were observed as a result of ALAD inhibition by Pb, Cd and especially DA, which led to an accumulation of δ-ALA in tissues. The accumulation of δ-ALA observed following exogenous application of ALA or by the presence of a specific inhibitor of ALAD (DA) did not correlate with antioxidants (tocopherols, SOD and enzymes of the Halliwell-Asada cycle) induction and photoprotective pigments (carotenoids) content. In fact, a higher antioxidant and photoprotective response in Pb treated plants was observed in spite of accumulating less δ-ALA. According to these results, we conclude that

the antioxidant and photoprotective responses observed in *L. minor* plants treated with Cd or Pb are not induced by δ-ALA accumulation.

8.2. Introduction

Metals are widespread contaminants that can occur as a consequence of natural processes, industrialization (metal smelting, motor vehicle transport, energy production) and modern agriculture (use of pesticides and fertilizers). They affect all living organisms and can generate chlorosis symptoms and oxidative stress in plants (Gonçalves et al., 2009; Gupta et al., 2009). To prevent this oxidative stress, plants protect photosynthetic apparatus from overexcitation through photoprotective systems, such as the xanthophylls cycle and they are able to cope with oxidative stress by scavenging ROS by antioxidant mechanisms (antioxidant enzymes and antioxidant compounds as ascorbate, glutathione and tocopherols).

Cadmium and lead are non-redox phytotoxic metals which can alter the cellular redox state of plants indirectly by interfering with components of the photosynthetic system (Becerril et al., 1989), mithocondrial respiratory electron chain and with the antioxidant defense system (Bi et al., 2009). Porphyrins biosynthetic pathway, which leads to chlorophyll, heme, siroheme and phytochromobilin synthesis in plants (Tanaka and Tanaka, 2007) and to heme in animals (Dailey, 1997), can be also inhibit by Cd (Padmaja et al., 1990) and Pb (Gupta et al., 2009).

Several studies with animals and human tissues (Rocha et al., 1995; Sakai, 2000) have described that the enzyme δ-aminolevulinic acid dehydratase (ALAD), an intermediate of the pathway which catalyzes the formation of porphobilinogen (PBG) from two molecules of δ-aminolevulinic acid (δ-ALA), is especially sensible to Pb. As a result of the enzyme inhibition, δ-ALA accumulates, and its autooxidation to 4,5-dioxovaleric acid generates radical

intermediates and ROS which lead to antioxidant system activation and finally may cause oxidative stress (Bechara et al., 2007).

In plants, it has also been described that Pb inhibits ALAD activity, leading to δ-ALA accumulation (Cenkci et al., 2010; Gupta et al., 2009) and a decrease in chlorophyll content (Becerril et al., 1988). However, it has not still been studied whether δ-ALA accumulation is responsible for the oxidative stress observed in plants exposed to this phytotoxic metal. On the other hand, several authors (Gonçalves et al., 2009; Noriega et al., 2007; Padmaja et al., 1990) have described an inhibition of ALAD activity in plants exposed to Cd. Noriega et al. (2007) suggested that δ-ALA accumulation as a result of ALAD inhibition by Cd was highly responsible for oxidative stress generated in soybean tissues, but further evidence is needed.

The aim of this study was to evaluate the effects of δ-ALA accumulation in plant tissues caused by inhibition of porphyrins pathway by Pb and Cd and its relationship with the antioxidant and photoprotective systems of *Lemna minor* L.

8.3. Materials and methods

8.3.1. Experimental design

Duckweed colonies (*Lemna minor* L.) (1.2 g) were placed in plastic containers with 0.5 l of Hoagland modified growth medium (Cross, 2002), renewed 2 days after the beginning of the experiment. Plants were grown in a controlled environment chamber at 25/18 ºC, 60/70% relative humidity, 14/10 h day/night photoperiod, and a photosynthetic photon flux density of 300 μmol photon m^{-2} s^{-1}. Plants were exposed for 4 days to (i) 5 μM of Cd^{2+} (Cd), (ii) 50 μM of Pb^{2+} (Pb), (iii) 100 μM of 4,6-dioxoheptanoic acid (DA) or (iv) 100 μM of δ-aminolevulinic acid (ALA). Growth media was supplemented with potassium nitrate to replace metal salts in the non-metal treatments and Geochem-EZ program was used to calculate the concentration of metals required to reach the above-mentioned free concentration in the growth medium. Control and each

treatment were conducted in triplicate. Plants material collected at the beginning and at the end (after 4 days) of the experiment was frozen in liquid nitrogen and stored at -80 ℃ until biochemical determination. R elative growth rate (RGR) of *L. minor* colonies was determined 2 and 4 days after treatments and calculated according to the formula [1], where W_1 and W_2 are plant fresh weights at times t_1 and t_2, respectively.

$$RGR = (\ln W_2 - \ln W_1)/(t_2 - t_1) \quad [1]$$

8.3.2. Physiological and biochemical determination

Concentration of δ-ALA was determined after Becerril et al. (1992). For ALAD (EC 4.2.1.24) extraction, the method of Naito et al. (1980) was used, and ALAD activity was determined according to Schneider (1970) with minor modifications: modified Ehrlich's reagent (Becerril et al., 1992) was mixed with a same volume of sample, and absorbance was spectrophotometrically determined at 555 nm after 10 min. A standard curve of PBG was used for ALAD activity calculation. Soluble protein was determined in ALAD homogenate according to Bradford (1976), using bovine serum albumin as a standard.

Photosynthetic pigments and tocopherols were extracted under dim light with a tissue-tearor at 10 000 rpm. Plants (40 mg FW) were homogenized with 1.5 ml of acetone (containing 0.5 g L^{-1} of $CaCO_3$). The homogenate was centrifuged at 16 100 g and 4 ºC for 20 min and the supernatant was filtered through a 0.2 µm syringe filter. Pigments and tocopherols were determined by reverse-phase HPLC according to García-Plazaola and Becerril (1999) with the modifications described by García-Plazaola and Becerril (2001).

Enzyme extraction was carried out following Polle et al. (1993). For ascorbate peroxidase (APX, EC 1.11.1.11) and monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) extraction, 10 mM of ascorbate was added to the extraction buffer (Polle et al., 1990). For superoxide dismutase (SOD, EC 1.15.1.1), APX and MDHAR activities determination, supernatants were

previously purified through PD SpinTrap G-25 microcolums which had been equilibrated with 20 mM of phosphate buffer pH 7.8 for SOD and 0.5 M of phosphate buffer pH 7 with 1 mM of ascorbate for APX and MDHAR.

The method described by for McCord and Fridovich (1969) was used for SOD activity determination. One unit of activity was defined as the amount of SOD required to decrease the rate of cytochrome C reduction by 50% at 25 ºC. Determination of APX, MDHAR, and glutathione reductase (EC 1.6.4.4) activities were determined according to Asada, (1984), Borraccino et al. (1989) and Wingsle and Hällgren (1993), respectively. Protein content of the enzymatic extracts was measured according to Lowry et al. (1951) and Peterson (1983).

8.3.3. Statistical analysis

Every group of data was checked for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Cochran C test) and, when possible, differences between treatment means were analyzed by one-way ANOVA followed by Duncan test ($p<0.05$). Differences among data not satisfying assumptions for ANOVA were analysed using t-Student test ($p<0.05$). All statistical analyses were carried out with the SPSS v. 19.0 software.

8.4. Results

A progressive reduction of relative growth rate in plants treated with Pb, Cd and DA was observed (Fig. 8.1). At the end of the experiment, DA and Pb treatments inhibited plants growth by 59% as compared to control, while Cd inhibited it by 50% (Fig. 8.1). On the opposite, ALA treatment did not affect significantly *L. minor* growth throughout the experiment (Fig. 8.1).

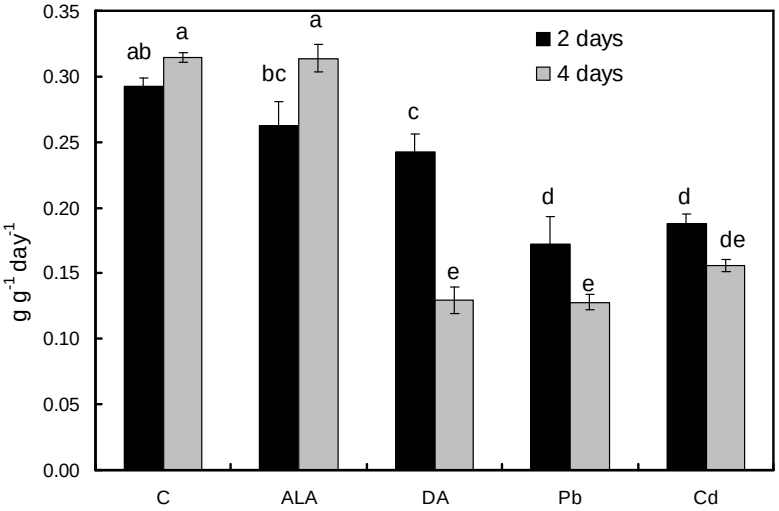

Fig. 8.1. Relative growth rate of *L. minor* 2 and 4 days after been exposed to the different treatments (mean ± SE). Different letters indicate statistically significant differences among treatments.

Lead and Cd inhibit ALAD activity of plants by 49% and 54%, respectively (Fig. 8.2a). The enzyme activity was inhibited by 88% in plants treated with DA, a competitive inhibitor of ALAD activity (Fig. 8.2a). On the other hand, no differences were observed in ALAD activity between control and ALA treated plants (Fig. 8.2a).

As a result of ALAD inhibition, the level of δ-ALA as compared with control level was 74-fold, 3.8-fold, 1.9-fold and 1.3-fold for DA, ALA, Pb and Cd, respectively (Fig. 8.2b).

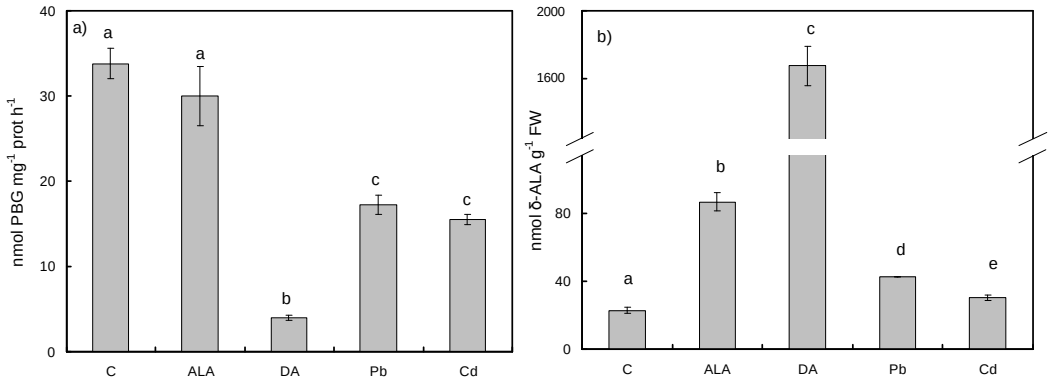

Fig. 8.2. ALAD activity (a) and δ-ALA concentration (b) of *L. minor* 4 days after been exposed to the different treatments (mean ± SE). Different letters indicate statistically significant differences among treatments.

After 4 days, chlorophyll content was only significantly reduced in DA and Cd treatment by a 50% and a 15%, respectively (Fig. 8.3a). In Pb and ALA treated plants no significant reduction of chlorophyll was detected (Fig. 8.3a). We observed an increase of zeaxanthin (Z) and a decrease of violaxanthin (V) levels in plants treated with the inhibitor or the metals as a result of V phototransformation to Z. Consequently, an increase in the de-epoxidation index (A+Z/VAZ, A: antheraxanthin) (Table 8.1) was observed in Pb, Cd and DA treatments, but it was only statistically significant for Pb. Total VAZ, neoxanthin, lutein and β-carotene content, and therefore, total carotenoids concentration (Table 8.1) significantly decreased in DA treated plants. Similarly, plants exposed to Cd experienced a decrease, although to a lower extent, in β-carotene and lutein levels, which was reflected in total carotenoids content (Table 8.1). ALA treated plants did not change their pigments content as compared with control (Table 8.1).

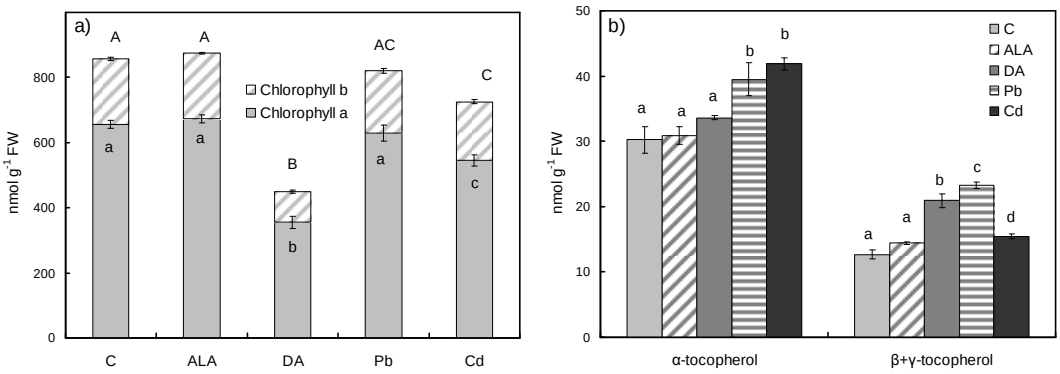

Fig. 8.3. Chlorophylls (a) and tocopherols (c) concentrations of *L. minor* plants 4 days after been exposed to the different treatments (mean ± SE). Different letters indicate statistically significant differences among treatments.

Plants accumulated α-tocopherol after exposition to metals, a 31% and 38% more than control plants for Pb and Cd, respectively (Fig. 8.3b). β+γ-tocopherol was accumulated in response to metals (Pb and Cd) and the ALAD inhibitor (DA) (Fig. 8.3b).

The activity of several antioxidant enzymes (SOD, APX, MDHAR and GR) is shown in Fig. 8.4. No significant differences were observed among treatments and control for SOD and MDHAR activities (Fig. 8.4a,b). Activity of APX was only higher than control in Pb treated plants (Fig. 8.4c) and GR activity increased significantly in plants exposed to metals and ALAD inhibitor (Fig. 8.4d).

Table 8.1. Carotenoids and de-epoxidation index (A+Z/VAZ rate) in *L. minor* plants 4 days after been exposed to the different treatments. Units: nmol g^{-1} PF (mean ± SE). Different letters indicate statistically significant differences among treatments.

	C	ALA	DA	Pb	Cd
Neoxanthin	33.5 ± 1.1 a	34.1 ± 0.6 a	20.2 ± 0.7 b	34.2 ± 1.7 a	30.9 ± 1.5 a
Lutein	111.7 ± 3.9 a	116.2 ± 1.5 a	79.1 ± 3.3 b	118.9 ± 6.0 a	99.1 ± 2.6 c
Violaxanthin (V)	55.3 ± 6.5 a	60.5 ± 0.3 a	39.3 ± 3.0 bc	36.2 ± 2.6 c	49.3 ± 4.5 ab
Antheraxanthin (A)	10.0 ± 2.6 a	8.8 ± 1.4 a	8.8 ± 0.6 a	15.1 ± 1.6 a	13.9 ± 1.4 a
Zeaxanthin (Z)	7.5 ± 0.6 a	7.9 ± 0.8 a	11.4 ± 0.8 ab	15.9 ± 3.3 b	10.0 ± 1.1 a
Total VAZ	72.7 ± 3.4 a	77.1 ± 2.2 a	59.5 ± 2.7 b	67.1 ± 3.9 a b	73.1 ± 2.3 a
A+Z / VAZ	0.245 ± 0.056 a	0.215 ± 0.021 a	0.341 ± 0.028 ab	0.457 ± 0.054 b	0.329 ± 0.042 ab
β-carotene	83.8 ± 1.8 a	85.9 ± 1.7 a	54.2 ± 3.1 b	80.2 ± 2.9 a	68.6 ± 2.0 c
Total carotenoids	301.7 ± 10.1 ab	313.3 ± 5.5 a	213.1 ± 9.6 c	300.3 ± 14.5 ab	271.7 ± 8.2 b

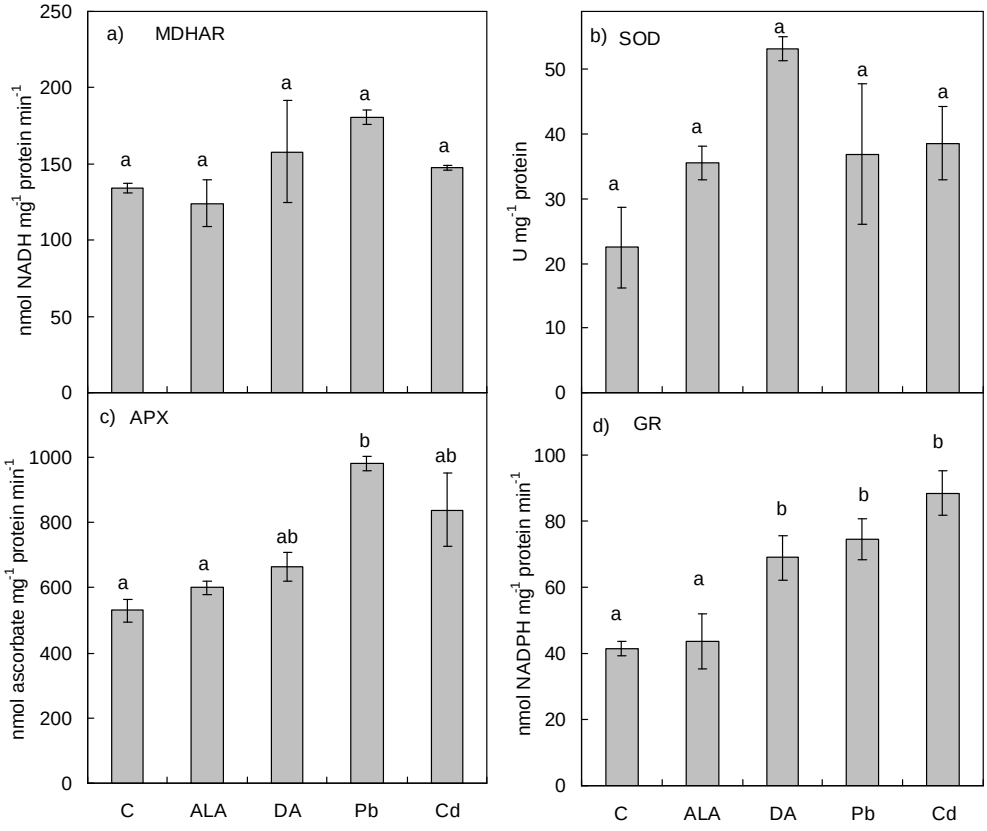

Fig. 8.4. Monodehydroascorbate reductase (a), superoxide dismutase (b), ascorbate peroxidase (c) and glutathione reductase (d) reductase of *L. minor* 4 days after been exposed to the different treatments (mean ± SE). Different letters indicate statistically significant differences among treatments.

8.5. Discussion

It has been widely described that metals can cause oxidative stress in plants (Sanità di Toppi and Gabbrielli, 1999; Singh et al., 1997). Some authors have suggested that this could be caused by the accumulation of δ-ALA, at least in Cd (Noriega et al., 2007). In this study, we assess the implications of δ-ALA

accumulation caused by Pb and Cd in the antioxidant and photoprotective responses of *L. minor* plants.

Porphyrins metabolism in plants is a highly regulated process to avoid the accumulation of photosensitizers. In fact, as a consequence of the inhibition of the porphyrin pathway by photodynamic herbicides some toxic photosensitizing intermediates accumulate causing oxidative stress and cell rupture (Becerril & Duke, 1989; Sherman et al., 1991). In this sense, high concentrations of exogenous δ-ALA have been used as herbicides (Rebeiz et al., 1990) because of the ability of this substrate to induce an overproduction of tetrapyrroles that could act as powerful photosensitizers by causing hydroxyl radicals involving deleterious effects on the photosynthetic apparatus (Härtel et al., 1996).

Plants treated with exogenous ALA presented a 3.8-fold higher concentration of internal δ-ALA than control plants (Fig. 8.2a) but they did not cause any phytotoxic effects on terms of growth (Fig. 8.1), ALAD inhibition (Fig. 8.2b), photosynthetic pigments content (Fig. 8.3a and Table 8.1), tocopherols levels (Fig. 8.3b) and antioxidant enzymes activity (Fig. 8.4). Other studies showed a similar tolerance of *Lemna pausicostata* L. to this intermediate (Becerril et al., 1992).

As a result of ALAD inhibition in DA, Pb and Cd treated plants, δ-ALA accumulates, especially in DA treatment (Fig. 8.2). Actually, an inverse relationship between ALAD inhibition and δ-ALA accumulation was observed. Similarly, Meller and Gassman (1981) found an accumulation of δ-ALA and a total inhibition of ALAD activity in barley leaves when treated with the inhibitor DA. Noriega et al. (2007) and Cenkci et al. (2010) also observed an accumulation of δ-ALA as a result of ALAD inhibition in fodder turnip and soybean plants exposed to Cd and Pb, respectively. Two factors could be responsible for δ-ALA accumulation in DA treatments: the effective inhibition of the enzyme and an interference with the feedback mechanism on δ-ALA synthesis Mg-chelatase mediated (Stenbaek and Jensen, 2010) and with other porphyrin intermediates.

The drastic inhibition of ALAD caused a significant reduction in total chlorophyll content (Fig. 8.3a) in DA treated plants. Similarly, Meller and Gassman (1981) observed an inhibition of approximately 40% in chlorophyll content of barley leaves when feeding with 5 mM DA. In spite of ALAD inhibition, chlorophyll content in plants exposed to Pb was not affected and Cd treated plants had a slightl, but significant decrease (15%) (Fig. 8.3a). As plant growth (i.e., new fronds development) was inhibited about 50% in metals and DA treatments, the phytotoxic effect on growth caused by metals were not directly related with inhibition of photosynthetic pigments.

Under non-stressful conditions, ROS are scavenged by antioxidative mechanisms (Foyer and Noctor, 2006) but this equilibrium can be disturbed by abiotic stresses, such as heavy metals, which involved an increase in intracellular levels of ROS. Accumulation of ROS as a consequence of phytotoxic metals can be counteracted by enzymatic antioxidants, non-enzymatic metabolites (such as tocopherols) and photoprotective molecules (such as carotenoids) which protect the photosynthetic apparatus. Carotenoids are photosynthetic accessory pigments, but also protective compounds preventing the overexcitation of the photochemical system. The decrease (29%) in total carotenoids content of DA treated plants (Table 8.1) is probably a response to adjust pigment composition of antennas to lower chlorophyll content (48%). In Cd treated plants, these pigments concentration also decreased, but to a lower extent (15% and 10% for chlorophylls and carotenoids, respectively). The diminution of chlorophylls content could be due not only to ALAD inhibition, but also to the pigments degradation. The β-carotene diminution observed in DA and Cd plants (Table 8.1) involved a lower antioxidant protection of plants as this carotenoid is an efficient scavenger of singlet molecular oxygen (Sies and Stahl, 1995). Studies with *Brachiaria decumbens* also reported that Cd exposure reduced β-carotene content in leaves (dos Santos et al., 2012). The increase of A+Z/VAZ ratio observed in Pb treatment was due to higher levels of A and Z, the main xanthophylls responsible for energy heat dissipation (Demmig-Adams and Adams, 1992; Gilmore and Yamamoto, 1993) and lower levels of V (Table 8.1).

The phototransformation of V to A and Z suggests an enhancement of photoprotection against the overexcitation of the photosynthetic apparatus which is associated with ROS generation (Janik et al., 2008).

Tocopherols protect membrane stability and provide a defense against oxidative stress through ROS and lipid peroxyl radicals scavenging. Treatments of DA and mainly Pb and Cd enhanced tocopherols synthesis (Fig. 8.3b). In a previous study, Artetxe et al. (2002) observed an induction of α-tocopherol level when *L. minor* plants were exposed to 50 ppm of Cd for 3, 6 and 9 days. Similarly, Zengin and Munzuroglu (2005) found an increase in α-tocopherol level when bean seedlings were exposed to increasing Pb concentrations for 10 days. Interestingly, a different response of tocopherol isomers synthesis in Pb and Cd treated plants was observed (Fig. 8.3b), and therefore, a deeper study is needed to understand the implication of the different tocopherol isomers in the antioxidant system of *L. minor* under different metal stresses.

The absence of relationship between δ-ALA accumulation and synthesis of antioxidant compounds (tocopherols) and increase of photoprotective response indicates that induction of oxidative stress is not directly related to the δ-ALA accumulation. In fact, higher levels of α-tocopherol (Fig. 8.3b), carotenoids and A+Z/VAZ (Table 8.1) in plants treated with Pb coincided with lower levels of δ-ALA accumulation (Fig. 8.2b), compared to DA treated plants.

In the present study, the activity of the enzyme SOD, responsible for superoxide anion reduction to the less toxic H_2O_2, was not affected by any treatment. Only in Pb treated plants APX activity increased (Fig. 8.4c), an enzyme which utilizes ascorbic acid as a specific electron donor to reduce H_2O_2 to water with the concomitant generation of monodehydroascorbate (MDHA). However, MDHAR, the enzyme for ascorbate regeneration, was not affected by any treatment. The increase of the activity of GR, an enzyme involved in reducing glutathione from its oxidized form, in DA, Pb and Cd treated plants (Fig. 8.2c) assured the maintenance of the glutathione pool, a non-enzymatic water soluble antioxidant essential for ROS scavenging (Jozefczak et al., 2012). In a

previous study with *L. minor* plants exposed to Cd, we observed a decrease in glutathione concomitant with an increase in phytochelatines which are synthesized from glutathione (Artetxe et al., 2002). Antioxidant response of plants exposed to Cd or Pb has been largely studied and it has been observed that antioxidant capacity depends on the plant species, the tissue exposed, the metal, its concentration and the exposure time. Other authors have found either increases or decreases in antioxidative enzymes (MDHAR, GR, APX, SOD, catalase and peroxidase) caused by Cd and Pb depending on the plant species, metal concentration and sampling time (Romero-Puertas et al., 2007; Razinger et al., 2008; Paczkowska et al., 2007, among others).

As shown before by tocopherols and carotenoids, the absence of relationship between δ-ALA accumulation and the increase of antioxidant activities (APX and GR) indicates that induction of oxidative stress is not directly related to δ-ALA accumulation. In fact, the increase of GR and APX activities in Pb treated plants was similar or higher, respectively, than in DA treated plants (Fig. 8.3c,d), coinciding with lower values of δ-ALA accumulation (Fig. 8.2b).

Increase of antioxidant enzymatic activities, tocopherols levels and de-epoxidation index seems to be the result of the stimulation of the antioxidant and photoprotective systems as a consequence of ROS production.

The observed difference in the way that *L. minor* responded to Pb and Cd toxicity (antioxidant and photoprotective pigments) suggests that, although the plants were exposed to metal concentrations that involved a similar growth inhibition, metal absorption, transport and subsequent localization and effects at cellular level were dependent on the metal. In fact, metal toxicity relies on these processes.

Plants treated with DA, which accumulated 39-57 times more δ-ALA than Pb and Cd treatments (Fig 8.2b), respectively, underwent an increase in tocopherols (Fig. 8.3b) and antioxidant activities (Fig. 8.4) similar or lower to metals treated plants. Moreover, the higher value of the de-epoxidation index (A+Z/VAZ) in Pb treated plants (Table 8.1) indicates a higher activation of the

photoprotective system mediated by the xanthophylls cycle pigments in these plants. In addition, no differences in the determined antioxidants or in the photoprotective system were observed in plants exposed to exogenous δ-ALA in spite of being δ-ALA accumulation in the plant greater than in metal treated plants. The different response to Pb in plants and animal could be a consequence of organelle location of the tetrapyrrole biosynthetic pathway: mitochondria and cytosol in animals (Bechara et al., 2007) and mainly plastids in plants (Tanaka and Tanaka, 2007). Even though it has been previously suggested that δ-ALA accumulation in soybean tissues because of Cd could be responsible for oxidative stress in soybean plants (Noriega et al., 2007), our results indicated that δ-ALA accumulation was not mainly responsible for early antioxidant and photoprotective system response when *L. minor* was exposed to Cd and Pb. Other harmful mechanisms such as activation of pro-oxidative enzymes or interference with components of essential physiological processes could be the responsible mechanisms for ROS generation in the plant when exposed to phytotoxic metals.

Further studies are needed to understand the early steps of heavy metals toxicity which cause oxidative stress.

9. General Discussion

9. Discusión General

El objetivo que persigue un proceso de fitoestabilización de metales consiste en disminuir la biodisponibilidad de los metales en el suelo y recuperar su salud. En concreto, la técnica de quimiofitoestabilización o quimioestabilización asistida con plantas busca complementar la estabilización química producida gracias a la adición de enmiendas con el efecto adicional que produce el crecimiento de la vegetación. Se trata, por tanto, de optimizar el uso de enmiendas orgánicas o inorgánicas buscando reducir la biodisponibilidad de los metales (estabilización química); así como favorecer el crecimiento de una vegetación (generalmente formada por especies exclusoras) capaz de inmovilizar los metales en su rizosfera, contribuyendo así a la reducción de la (bio)disponibilidad de estos contaminantes. El objetivo es evitar o limitar la movilización del contaminante en el suelo, restringiendo así la afección que supone la movilización y bioacumulación de estos metales a través de su paso a la cadena trófica y su lixiviación o afección de aguas subsuperficiales y subterráneas.

En esta tesis doctoral se han realizado varios estudios sobre un suelo procedente de un entorno minero, tanto en microcosmos como en campo, que han sido organizados en distintos capítulos (CAPITULOS 4 a 7). Para estudiar la efectividad de enmiendas orgánicas en la estabilización de los metales y las condiciones fisicoquímicas y biológicas del suelo, se realizó inicialmente un ensayo con cuatro enmiendas orgánicas (gallinaza, purín bovino, estiércol de oveja y lodos de papelera mezclados con gallinaza) en condiciones controladas de microcosmos (CAPITULO 4). En el siguiente capítulo (CAPITULO 5) se estudió, bajo condiciones controladas, el efecto conjunto de la aplicación de enmiendas orgánicas con la plantación de la especie nativa del entorno minero *Festuca rubra*, para indagar en la contribución de la vegetación en la fitoestabilización. En el CAPITULO 6 se llevó a cabo un ensayo de fitoestabilización en un entorno afectado por la minería de Pb y Zn en Carranza (Bizkaia), donde se aplicaron distintas enmiendas orgánicas en zonas vegetadas

y no vegetadas. En este estudio, se revegetaron además las zonas no vegetadas con explantes de *F. rubra* procedentes del mismo entorno minero previamente propagadas en invernadero. A lo largo de estos tres capítulos (4, 5 y 6), se emplearon diversos indicadores fisicoquímicos y biológicos (microbianos y vegetales) de salud del suelo, con el fin de determinar su idoneidad para la monitorización de procesos de fitoestabilización asistida con enmiendas orgánicas. También se estudió la contribución de las poblaciones microbianas presentes en las enmiendas sobre la biodiversidad funcional microbiana del suelo (CAPITULOS 6 y 7) y la abundancia génica de bacterias y hongos (CAPITULO 7). Ya que algunos biomarcadores vegetales son buenos indicadores para determinar la ecotoxicidad del suelo mediante bioensayos, se investigó la inhibición de la ruta de las porfirinas por los metales Pb y Cd y su relación con el estrés oxidativo y sistemas de fotoprotección en la especie modelo *Lemna minor*.

Si bien existe una serie de trabajos publicados sobre estabilización química en suelos, el proceso de estabilización de las enmiendas y su efecto sobre la biodisponibilidad y la salud del suelo no han sido aún suficientemente estudiados, en particular en lo referente a los efectos a corto plazo. Asimismo, existe un amplio desconocimiento del efecto que la población microbiana de las enmiendas puede tener sobre la diversidad funcional de las comunidades microbianas del suelo. Con el fin de dar respuesta a estas dos cuestiones y de seleccionar las enmiendas orgánicas más eficaces para la estabilización química de un suelo minero contaminado principalmente con Pb y Zn, se llevó a cabo un estudio en microcosmos durante un mes con cuatro enmiendas orgánicas (CAPITULO 4). En este estudio se determinaron una batería de parámetros químicos y microbiológicos indicadores de la salud del suelo, y un indicador de fitotoxicidad (bioensayo de elongación radical), con el fin de evaluar la idoneidad de estos marcadores para la monitorización del proceso de estabilización química en la recuperación de la salud del suelo. Tanto el estiércol de oveja como los lodos de papelera con gallinaza fueron los más efectivos para la disminución de la biodisponibilidad de Pb y Zn en el suelo, incrementando la

elongación radicular en el bioensayo de lechuga. El test de elongación radical es un excelente ensayo para evaluar la biodisponibilidad de metales y ecotoxicidad del suelo por su sensibilidad, a la par que sencillo y barato. La modificación del pH por las enmiendas es un factor crucial para la reducción de la biodisponibilidad de metales.

También se observó que los microorganismos de las enmiendas no afectaban a la diversidad funcional de las poblaciones microbianas nativas del suelo (Biolog EcoPlate^{TM}), excepto en los lodos con gallinaza, donde el proceso de esterilización de la enmienda en autoclave incrementó el consumo de algunos sustratos. A pesar de que parte del aumento en la actividad de los microorganismos del suelo causado por las enmiendas desaparece al cabo de un mes, la gallinaza y el purín vacuno resultaron ser las enmiendas que más estimularon la actividad microbiana. Estas variaciones temporales en los efectos de las enmiendas indicaron una gran relevancia de la materia orgánica aportada por las enmiendas en el estímulo de la actividad microbiana de un suelo. Como conclusión, podemos indicar que el estímulo de la actividad microbiana se debió más al aporte de la materia orgánica asimilable, que al beneficio que supone la reducción de la biodisponibilidad de los metales. En los estudios de fitoestabilización con enmiendas orgánicas es muy importante, para monitorizar la ecotoxicidad del suelo, la utilización de otros taxones más independientes del aporte orgánico, como el test de elongación radical.

El uso de especies vegetales exclusoras es la base de la fitoestablización ya que estas plantas son capaces de inmovilizar los metales sin necesidad de extraerlos del suelo a través de mecanismos de absorción, acumulación y adsorción en las raíces y precipitación en la rizosfera (Baker, 1981). La especie exclusora *Festuca rubra* era la especie dominante en el entorno minero de estudio (Epelde y cols., 2010; Barrutia y cols., 2011). Para evaluar la eficacia de las enmiendas orgánicas antes de su aplicación en el campo se realizó un estudio previo de fitoestabilización asistida del suelo minero, en microcosmos (CAPITULO 5). Para ello, en las macetas del ensayo anterior de estabilización química, se crecieron plantas de la especie *Festuca rubra* durante 18 semanas.

Todas las enmiendas produjeron una mejora sustancial al final del estudio sobre los parámetros fisicoquímicos y biológicos pero con algunas particularidades propias de cada tratamiento. El estiércol de oveja y los lodos de papelera con gallinaza fueron los tratamientos más efectivos para reducir la biodisponibildad y fitodisponibilidad de metales, mientras que la gallinaza tuvo un efecto estimulatorio de la actividad biológica (plantas y microorganismos) a pesar de su mayor biodisponibilidad de metales. Teniendo en cuenta todos estos factores podemos indicar que el estiércol de oveja fue la mejor enmienda para la fitoestabilización asistida del suelo minero ya que combinaba una disminución del metal biodisponible en el suelo y del contenido en metal de la planta, con un efecto favorable sobre el crecimiento de la planta y en la actividad y biomasa de los microorganismos del suelo. La simple presencia de la planta en el control (sin enmienda) no contribuye a la mejora de los parámetros del suelo. Este aspecto es muy relevante ya que aunque la plantación de *F. rubra* es una buena opción para la fitoestabilización de entornos mineros, resulta necesaria la fertilización orgánica del suelo para la fitoestabilización a corto plazo de un suelo minero altamente contaminado con metales.

Finalmente, se realizó un estudio de fitostabilización asistida en campo evaluando las enmiendas de purín vacuno, gallinaza y lodos de papelera con gallinaza. El estiércol de oveja, que resultó un tratamiento interesante, no se pudo evaluar al no poder garantizar su trazabilidad (composición y compostaje). Las enmiendas fueron aplicadas en cuatro emplazamientos (dos vegetados y dos no vegetados) de la zona minera de estudio con el fin de evaluar la efectividad de estas enmiendas en la fitoestabilización de estos suelos en condiciones naturales de campo (CAPITULO 6). Además, se evaluó el potencial de revegetación de *Festuca rubra* en los suelos enmendados inicialmente desprovistos de vegetación.

En general, se observaron mejoras más importantes en las zonas inicialmente no vegetadas, y en aquellas zonas cuya toxicidad inicial era mayor. Tal y como habíamos observado previamente en los ensayos en microcosmos (CAPITULOS 4 y 5), las enmiendas favorecieron una disminución del Pb y Zn

biodisponible a la vez que incrementan la materia orgánica y los nutrientes del suelo. Estos efectos favorecieron conjuntamente el crecimiento de la vegetación y la mejora de los parámetros de los microorganismos del suelo. La gallinaza fue la enmienda que causó mayor beneficio en el crecimiento de *F. rubra* en las zonas no vegetadas, tal como habíamos visto en el ensayo de fitoestabilización asistida en microcosmos (CAPITULO 5). Por otro lado, tanto los lodos de papelera con gallinaza (al igual que habíamos comprobado previamente en el CAPITULO 5) como el purín bovino favorecieron la disminución del contenido en metales de las hojas de *F. rubra* de las zonas vegetadas en mayor proporción que los otros tratamientos. Dos meses después de la aplicación de la gallinaza, el efecto sobre el incremento de la biomasa vegetal fue más destacado, pero disminuyó a los seis meses probablemente debido al agotamiento progresivo de los nutrientes aportados por las enmiendas, lo cual redujo la tasa de crecimiento de las plantas.

En cuanto a los parámetros químicos, los lodos con gallinaza y el purín bovino fueron las enmiendas que más disminuyeron la biodisponibilidad de metales del suelo en las zonas no vegetadas y vegetadas, respectivamente. Por otra parte mientras que las enmiendas favorecieron un incremento del pH del suelo en los ensayos en microcosmos (CAPITULOS 4 y 5), contribuyendo a la inmovilización del Pb y el Zn en el suelo, en el ensayo de campo (CAPITULO 6) las enmiendas no modificaron significativamente el pH del suelo. Diferencias en las dosis y en el modo de aplicación de las enmiendas podrían explicar este hecho. Sin embargo, y sin tener en cuenta las enmiendas, en el suelo de la mina si existió una relación inversa entre el incremento de pH y la disponibilidad de metales, así por ejemplo en la zona más contaminada (SITIO 4) se observó una menor biodisponibilidad de metales en el suelo, a pesar de presentar las mayores concentraciones pseudo-totales de metales, debido un pH más elevado.

La aplicación de las enmiendas provocó un aumento mayor en la biomasa microbiana (determinada como respiración inducida) que en su actividad (determinada como respiración basal). Este aspecto es interesante ya que en el ensayo en microcosmos (CAPITULO 7) determinamos una mayor abundancia de

bacterias y hongos (mediante incremento de copias de 16S rRNA bacteriano y 18 rRNA fúngico) en los suelos enmendados. Los cambios observados hacia valores más altos en la relación hongos:bacterias del suelo, podrían estar relacionados con un aumento de los ciclos de nutrientes más lentos y conservativos propios de los hongos. En general, el purín bovino fue la enmienda que más favoreció la actividad microbiana, según lo indicaron los parámetros microbianos medidos, resultado que coincide con lo observado en los ensayos en microcosmos (CAPITULOS 4 y 5). Asimismo, de manera general, el Zn fue el metal que más condicionó la diversidad funcional de los microorganismos edáficos (CAPITULO 7).

Un aspecto relevante de esta memoria es la utilización y validación de los indicadores biológicos (parámetros vegetales y microbianos), complementarios a los parámetros fisicoquímicos, para la evaluación de procesos de fitoestabilización asistida y la recuperación de la salud del suelo. Así, nuestros resultados enfatizan la importancia de monitorizar simultáneamente los parámetros químicos, los parámetros microbianos del suelo y los parámetros vegetales para evaluar correctamente los efectos producidos durante los procesos de fitoestabilización asistida. La gran variación en los parámetros estudiados y la información específica que aporta cada uno de ellos, indica que resulta imprescindible un programa de monitorización simultáneo y predefinido para evaluar correctamente la efectividad de los procesos de fitoestabilización asistida y establecer las necesarias correcciones sobre los tratamientos o aplicaciones de enmiendas. Como se ha indicado, los resultados de nuestro estudio avalan la utilización de los bioensayos de elongación radicular con *L. sativa* como una excelente metodología para evaluar la biodisponibilidad de metales y la fitotoxicdad potencial del suelo en procesos de fitoestabilización en microcosmos y en campo. En este sentido, es importante explorar nuevos biomarcadores vegetales de exposición, que incorporados a bioensayos, nos permitan la detección y evaluación de la contaminación del suelo por metales. Los metales pesados provocan estrés oxidativo alterando varios procesos fisiológicos y el contenido en metabolitos vegetales que pueden convertirse en

biomarcadores de estrés. Sin embargo, su modo de acción preciso no es aún bien conocido. Recientemente se ha relacionado en tejidos animales y humanos que la conocida inhibición de la enzima ácido δ-aminolevulínico dehidratasa (ALAD) por el Pb causa una acumulación de ácido δ-aminolevulínico (δ-ALA), cuyos productos de degradación generan EROs que provocan estrés oxidativo. Noriega y cols. (2007) sugirieron que la acumulación de ALA en plantas causada por Cd sería el responsable del estrés oxidativo observado en plantas de soja. Al estudiar el modo de acción del Pb y el Cd en la biosíntesis de profirinas y su respuesta antioxidante, así como la posible utilidad de algún metabolito como biomarcador de exposición a metales, en el CAPITULO 8 hemos probado que: (i) la respuesta fotoprotectora y antioxidante de plantas de *Lemna minor* expuestas a Cd o Pb no está directamente provocada con la acumulación de δ-ALA; (ii) los niveles de tocoferol aumentan siendo un biomarcador de potencial interés. Aún queda por determinar cuál o cuáles son los posibles mecanismos iniciales que desencadenan el estrés oxidativo en plantas en presencia de Cd y Pb y su diferencia con tejidos animales.

Como conclusión del estudio podemos indicar que antes de llevar a cabo un proyecto de fitoestabilización asistida en campo, es necesario realizar ensayos en microcosmos para evaluar las interacciones suelo-enmienda-planta, estableciendo las condiciones óptimas y evitando las posibles limitaciones del proceso fitoestabilizador. Para su correcta consecución en campo resulta imprescindible conocer no solo la especiación y concentración de los contaminantes, sino también los condicionantes edáficos y climáticos, y las propiedades fisicoquímicas y biológicas del suelo. En cualquier caso, los resultados obtenidos en microcosmos no son totalmente extrapolables a las condiciones de campo y nuestros resultados indican que las condiciones ambientales prefijadas de un estudio en microcosmos pueden alterar algunos parámetros esenciales del proceso de evaluación y selección de enmiendas.

Si bien el efecto beneficioso de las enmiendas sobre la actividad microbiana del suelo resulta mayor en las zonas no vegetadas en comparación con las vegetadas, el beneficio alcanzado por los suelos sin vegetación no es

suficiente para alcanzar los valores que muestran los parámetros biológicos iniciales en las zonas vegetadas. La aplicación de enmiendas orgánicas en procesos de fitoestabilización: (i) mejora sustancialmente los parámetros fisicoquímicos y biológicos del suelo; (ii) reduce la biodisponibilidad, bioacumulación y dispersión de contaminantes; (ii) favorece el crecimiento y tolerancia de la vegetación establecida, y (iv) estimula la colonización de los suelos desnudos, reduciendo la erosión de los mismos. Sin embargo, la gran heterogeneidad de los entornos mineros es uno de los principales problemas para la rehabilitación de este tipo de suelos y explica la respuesta diferencial de las distintas áreas expuestas al mismo tratamiento.

10. Final Conclusions

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1. The application of organic amendments to soil causes an increase in pH, organic matter and nutrient content, causing a decrease in metal bioavailability and plant phytotoxicity and an increase in soil microbial properties.
2. SHEEP and PAPER are more effective for reducing soil metal bioavailability, while poultry litter and cow slurry are most suitable for stimulation of microbiological processes in soil.
3. Organic amendments can be applied without a previous sterilization, as microbial populations present in amendments do not modify the native soil microbial functional diversity, except in a minor extent in soils amended with PAPER.
4. Our results highlight the importance of simultaneously monitoring physicochemical and microbial soil properties and plant toxicity (through Lactuca sativa root elongation bioassay*)* to properly assess the early effects of soil-amendment interactions during chemical stabilization studies.
5. Organic fertilization is necessary for short-term recovery of highly contaminated metalliferous soils, as it increases soil pH, organic matter and nutrients content, decreasing toxic metals bioavailability, facilitating performance and growth of native plant and increasing soil microbial activity and biomass.
6. POULTRY is the best treatment for plant performance and microbial activity, although it results in higher levels of phytoavailable metals. SHEEP and PAPER are recommended for reducing soil metal bioavailability and metal accumulation in plant tissues, thereby reducing the risk of metals entering the food chain.

7. SHEEP can be recommended for aided phytostabilization as it combines a decrease of metal bioavailability and plant metal content with benefits for plant and microbial performance.
8. Amendment application increases soil organic matter and nutrient content, especially in initially non-vegetated soils, decreases metal bioavailability and facilitates plant colonization and the growth of native plants. All of them are suitable for aided phytostabilization of mine soils.
9. POULTRY is the best treatment for stimulating plant growth and colonization of bare soils. COW and PAPER are the best treatments to decrease bioavailability in vegetated and non-vegetated areas, respectively, and to reduce metal content in plants.
10. COW can be recommended for aided phytostabilization as it combines a decrease of metal bioavailability and plant metal content with benefits for plant and microbial performance on vegetated and non-vegetated soils
11. Mine soil heterogeneity is one of the main problems to rehabilitate mine soils and explains the different response of different sites to the same organic amendments.
12. *Lactuca sativa* root elongation bioassay is a good bioindicator to assess soil metal bioavailability in phytostabilization processes under microcosm and field conditions.
13. Cd and Pb inhibit porphyrins pathway through the inhibition of δ-aminolevulinic acid dehidratase, causing an accumulation of δ-aminolevulinic acid in plant tissues.
14. Antioxidant and photoprotective response of Lemna minor plants exposed to Cd or Pb is not directly related to δ-aminolevulinic acid accumulation.
15. Tocopherol levels are biomarkers of potential interest to determine metal phytotoxicity, as they increase rapidly when plants are exposed to Cd and Pb.

11. References

11. References

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Dicen que en la vida hay que hacer tres cosas para que tu huella perdure en el mundo: plantar un árbol, escribir un libro y tener un hijo. De momento, ya he cumplido con las dos primeras…