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The importance of autochthonous microbial
communities in the sustainability of
vegetation for the phytomanagement of
semiarid mine tailings

Doctorado en Técnicas Avanzadas en Investigación y
Desarrollo Agrario y Alimentario (TAIDA)

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*“Cambiar el mundo, amigo Sancho,
no es ni utopía ni locura, es justicia”*

El Don Quijote de la Mancha

*A mis padres,
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Resumen

Los depósitos de estériles mineros (o balsas mineras) se encuentran formados por los residuos procedentes de las labores de concentración de minerales, y están considerados como uno de los principales problemas medioambientales asociados a las antiguas zonas de minería metálica. Los materiales que constituyen estas estructuras suelen presentar propiedades físico-químicas extremas como pHs ácidos o básicos, ausencia de estructura edáfica, salinidad, elevadas concentraciones de metal(oid)es, bajos contenidos en nutrientes y en materia orgánica, etc. Estas condiciones dificultan el establecimiento y crecimiento de plantas, manteniendo sus superficies desnudas, expuestas a los agentes erosivos. El empleo del fitomanejo, en términos de fitoestabilización, se considera la alternativa más eficiente para disminuir los riesgos medioambientales asociados a las balsas mineras. Esta técnica plantea la implantación de una cubierta vegetal estable sobre la superficie de las balsas mineras que evite la erosión y fije las partículas metálicas en la rizosfera. Con el fin de mejorar las deficientes condiciones de fertilidad de las balsas mineras y facilitar el establecimiento de la vegetación se recurre, de modo habitual, a la adición de enmiendas orgánicas. Si bien se ha constatado el efecto positivo que a corto plazo tiene la adición de estas enmiendas sobre la cubierta vegetal, no existe mucha información en relación al mantenimiento de la funcionalidad edáfica en estas balsas mineras en una escala temporal más amplia. Este hecho podría estar condicionado, en gran medida, por la presencia de una comunidad microbiana tolerante a las condiciones físico-químicas de los depósitos mineros, y que además, participe activamente en los procesos biogeoquímicos del suelo. La presente Tesis Doctoral pretende contribuir al avance del conocimiento de la relación entre los factores edáficos y microbiológicos implicados en el establecimiento de especies vegetales en antiguos depósitos mineros. Para ello, plantea como finalidad principal profundizar en el conocimiento de los procesos de evolución edáfica que dotan de sostenibilidad a los proyectos de fitomanejo de residuos mineros dentro del contexto de zonas áridas y semiáridas, evaluando la regeneración de la funcionalidad del suelo a través de la mejora de los ciclos biogeoquímicos y valorando los efectos concretos de la adición de enmiendas desde el punto de vista microbiológico. Para tal fin, la Tesis Doctoral fue dividida en dos partes: una fase de trabajo de campo en un depósito minero situado en el antiguo Distrito Minero de Cartagena-La Unión, y una fase experimental llevada a cabo en condiciones controladas en una cámara de cultivo situada en el Instituto de Biotecnología Vegetal de la UPCT. Los trabajos desarrollados en estas dos partes respondieron a cuatro objetivos específicos:

El primer objetivo consistió en determinar qué factores, de entre los aspectos edáficos, vegetales y microbiológicos, resultaban determinantes para la funcionalidad del suelo en el fitomanejo de balsas mineras en zonas semiáridas. Para ello, se realizó un estudio de campo utilizando un diseño experimental basado en un transecto a lo largo de una antigua balsa minera del Distrito de

Cartagena-La Unión. Dicho transecto incluyó tanto zonas no impactadas (control) como zonas dentro del cuerpo de la propia balsa en las que se tomaron muestras de suelo para su caracterización química y microbiológica. Los resultados mostraron que los cambios en las abundancias relativas de las bacterias a través del transecto se explicaron mejor por factores relacionados con la fertilidad del suelo y la presencia de plantas que por las concentraciones de metales. Esta presencia de vegetación espontánea en las balsas mineras permitió la transición desde órdenes de bacterias litotróficas, dominantes en áreas desprovista de vegetación, a grupos de carácter organotrófico, algunos de los cuales también estuvieron presentes en suelos no contaminados de la zona. Este último hecho permitió afirmar que los procesos de sucesión edáfica mediados por plantas podrían estimular ciclos biogeoquímicos similares a los que tienen lugar en sistemas no contaminados. A diferencia de las bacterias, las variaciones en la abundancia relativa de los hongos a través del transecto no se explicaron por los parámetros edáficos o la presencia de plantas, sino que parecían estar más relacionados con la distribución espacial o los tipos de sustratos orgánicos presentes.

El segundo objetivo se centró en identificar los factores edáficos, incluidos los microbiológicos, que determinaban el establecimiento de plantas con diferentes estrategias de crecimiento en depósitos mineros de entornos semiáridos. Para este fin, se realizó una caracterización edáfica y microbiológica de la rizosfera de una especie ruderal pionera, *Zygophyllum fabago*, y dos especies arbóreas, *Pinus halepensis* y *Tetraclinis articulata*, que crecen espontáneamente en la balsa minera seleccionada para el primer objetivo y trabajo de la Tesis. Los resultados revelaron que *Z. fabago* colonizó selectivamente zonas de la balsa caracterizadas por una alta salinidad (3.5 dS m^{-1}) y altos porcentajes de limo (42 %), mostrando una composición microbiana en su rizosfera que incluía grupos halotolerantes. Las dos especies arbóreas crecieron en áreas de salinidad moderada de la balsa (1.7 dS m^{-1}) con altos porcentajes de arena (85 %), donde *Actinomycetales* fue el orden bacteriano más abundante. Las diferencias en el comportamiento micorrícico de ambas especies arbóreas (ecto-micorrícico para *P. halepensis* y endo-micorrícico para *T. articulata*) podría explicar las diferencias encontradas entre su composición fúngica. En relación al fitomanejo, estos resultados mostraron que la colonización selectiva en nichos edáficos específicos de especies vegetales con diferentes estrategias de crecimiento permitiría aumentar la biodiversidad y la resistencia del sistema frente a factores de estrés ambientales.

El tercer objetivo se centró en evaluar los efectos de la aplicación de dos enmiendas orgánicas en la composición microbiana (bacteriana y fúngica) de balsas mineras. Para ello, se llevó a cabo un experimento en mesocosmos, donde un residuo minero procedente de la balsa caracterizada en el primer objetivo fue enmendado con compost de estiércol, biochar o su combinación. Estos tratamientos, junto a un tratamiento control sin enmendar, fueron mantenidos durante 6 meses en una cámara de cultivo con condiciones controladas. Se llevaron a cabo tres muestreos de suelo,

para su caracterización edáfica y microbiológica, en los meses uno, tres y seis. Los resultados mostraron que el compost tuvo un efecto más marcado sobre las propiedades de suelo que el biochar, especialmente en la etapa inicial del experimento donde los tratamientos con compost presentaron un mayor aumento de los valores de carbono orgánico disuelto y metales extraíbles en agua. Sin embargo, las concentraciones de estos parámetros disminuyeron a lo largo del experimento. En cuanto a la composición bacteriana, se observó una fuerte correlación de las abundancias relativas de algunos órdenes de bacterias (*Flavobacteriales* y *Sphingobacteriales*) con las concentraciones decrecientes de carbono lábil y la aparición progresiva conforme avanzaba el experimento de otros grupos más especializados en la descomposición de fuentes de carbono más estable (*Xanthomonadales* y *Myxococcales*). El biochar favoreció la presencia de bacterias descomponedoras (*Actinomycetales*) especializadas en la degradación de compuestos con lignina u otras sustancias de carbono recalcitrantes. A diferencia de las bacterias, solo unos pocos órdenes de hongos (*Sordariales* y *Microascales*) aumentaron su abundancia relativa en los tratamientos que contenían compost, mientras que el resto mostró una disminución o ningún efecto. El tratamiento combinado de biochar-compost podría resultar una opción viable para optimizar la funcionalidad microbiológica de los depósitos mineros debido a su capacidad para favorecer tanto microorganismos especializados en la degradación de materia orgánica lábil como compleja.

Por último, el cuarto objetivo tuvo la finalidad de evaluar los efectos de la presencia de plantas u hojarasca sobre la composición microbiana en sustratos mineros previamente enmendados con compost y/o biochar. Esto permitiría determinar la viabilidad de las comunidades microbianas presentes en residuos mineros enmendados para sustentar los ciclos biogeoquímicos desencadenados por la presencia de vegetación. Para ello, se seleccionó una especie vegetal pionera, *Piptatherum miliaceum*, procedente de depósitos mineros del Distrito Minero Cartagena-La Unión. Las semillas de *P. miliaceum* o su hojarasca se incorporaron a los mesocosmos empleados en el experimento recogido en el tercer objetivo. Los resultados mostraron que los efectos de la presencia de hojarasca o planta en la composición microbiana estuvieron muy condicionados por la presencia previa de enmiendas, especialmente de compost. En aquellos tratamientos que contenían compost el mayor crecimiento de las plantas favoreció una mayor diferenciación de la composición bacteriana y fúngica, mientras que en los tratamientos sin enmiendas o solo tratados con biochar (con menor crecimiento de plantas), la hojarasca jugó un papel más importante en la determinación de la composición microbiana, especialmente de bacterias. El mayor crecimiento de las plantas favoreció el incremento de grupos bacterianos cuyas abundancias relativas se habían visto disminuidas por la presencia de compost (e.g. *Actinomycetales*) y de otros grupos relacionados con el desarrollo rizosférico (e.g. *Rhizobiales*). Esto mostró la especial habilidad de las plantas para configurar su microbioma específico. El

empleo de combinaciones biochar-compost podría resultar viable para facilitar la consecución de una cubierta vegetal estable en depósitos mineros, ya que favorece el crecimiento vegetal (mayor biomasa), aporta una fuente de carbono estable (biochar) y facilita el desarrollo del microbioma rizosférico.

Abstract

Mine tailings are wastes composed by the left-overs of the ore-processing activities, and they are considered as the main responsible for environmental health impacts in former metallic mining areas. The bare areas of the tailings are usually impacted by wind and water erosion, which may spread the metal(loid) enriched particles to their surroundings. Phytomanagement by phytostabilization is considered a suitable alternative to decrease the environmental risks associated to mine tailings. This technique relies on the generation of a stable vegetation cover at the tailings surfaces to decrease the erosion by fixing the soil. However, soil conditions at tailings usually result unfavourable for plant growth (e.g. high metal(loid) concentrations, low fertility, high salinity) and soil conditioners are proposed to overcome this issue. Although there is a general acceptance of the benefits of using amendments for improving plant growth, there is little information on the sustainment of edaphic functionality at long-term scenarios. This issue could be related to the presence of a feasible microbiological community, which tolerates tailings conditions and actively supports biogeochemical processes. The general purpose of this PhD Thesis was to increase knowledge on the relationship between edaphic and microbiological factors involved in the spontaneous plant colonisation of mine tailings. This will improve the feasibility of phytomanagement in terms of phytostabilization applied to these environments in a semiarid climate context. In order to achieve this goal, the PhD thesis has been divided in two parts corresponding to a field survey data and mesocosm experiments carried out at the climate chamber facility at the IBV-UPCT. These research studies were designed to answer four specific goals:

The first goal was to elucidate which factors, including edaphic, vegetation and microbiological aspects, determine soil functionality in the phytomanagement of mine tailing piles in semiarid areas. For this purpose, a field study was conducted using an experimental transect design, which included a tailing pile and a non-mining impacted zone (as control) located at southeast Spain. The changes in bacterial relative abundances through the transect were better explained by soil fertility related factors (which in turn were related to the presence of plants) than by metals concentrations. The presence of vegetation at the tailings allowed the transition from lithotrophic bacterial orders, which dominated in bare tailings areas, to organotrophic oriented taxa, some of them shared with the non-polluted samples. These results seem to indicate that natural plant mediated successional processes might stimulate biogeochemical cycles similar to those occurring at non-polluted systems. This functionality of the impacted sites, which include unfavourable edaphic conditions, should be taken into account in the phytomanagement of mine tailings, since it may support its long-term sustainability. Unlike bacteria, variations in fungal relative abundances through the transect were not clearly explained by soil parameters or the

presence of plants, since they seem to be more determinant by spatial distribution or the type of organic substrates.

The second goal was focused on identifying the edaphic factors, including microbiological, which affect the establishment of plant species with contrasting growth patterns during the phytomanagement of a mine tailings pile. For this purpose, a comprehensive rhizosphere characterization was performed in an early ruderal colonizer, *Zygophyllum fabago* and two late successional tree species, *Pinus halepensis* and *Tetraclinis articulata*, growing at a mine tailings pile in southeast Spain. Results revealed that *Z. fabago* selectively colonized tailings patches characterized by high salinity (3.5 dS m⁻¹) and high silt percentages (42 %), showing a specific halotolerant rhizospheric microbial composition. The two tree species grew at moderate salinity areas of the tailings pile (1.7 dS m⁻¹) with high sand percentages (85 %) where *Actinomycetales* was the most abundant bacterial order (>10 % abundance). The contrasting mycorrhizal behaviour of both tree species (ecto-mycorrhizal for *P. halepensis* and endo-mycorrhizal for *T. articulata*) could explain the differences found between their fungal rhizospheric composition. In terms of phytomanagement, the selective plant species colonization following specific soil patches at mine tailings would increase their biodiversity and resilience against environmental stressors.

The third goal was to assess the effect of the application of two organic amendments, manure compost and biochar, on soil bacterial and fungal composition at metal(loid) enriched mine tailings. The addition of compost caused stronger effects on most of soil parameters and microbial composition than the biochar, especially at the initial stage of the experiment. However, the higher dependence on labile organic carbon for some bacterial groups at the treatments containing compost determined their decay along time (*Flavobacteriales* and *Sphingobacteriales*) and the appearance of other taxa more dependent on recalcitrant organic matter (*Xanthomonadales* and *Myxococcales*). Biochar favoured bacterial decomposers (*Actinomycetales*) specialized in high lignin substrates and other recalcitrant carbon compounds. Unlike bacteria, only a few fungal orders increased their relative abundances in the treatments containing compost (*Sordariales* and *Microascales*), while the rest showed a decrease or remained unchanged. The mix biochar-compost may result the best option to support a more diverse microbial population in terms of soil functionality that is able to decompose both labile and recalcitrant carbon compounds. This may favour the resilience of the system against environmental stressors.

Finally, the fourth goal was to evaluate the effects of the presence of plants or plant litter on the microbial composition in the previously amended tailings (with biochar and/or manure compost). This would allow to determine the feasibility of amendments for the long-term phytomanagement, as well as the ability or inability of the microbial community resulted from the amended tailings to support biogeochemical cycles without additional external inputs of amendments compatible

with the rhizospheric microbiome or litter. For this purpose, a pioneer plant species (*Piptatherum miliaceum*) of the former Cartagena-La Unión Mining District tailings was selected. *Piptatherum miliaceum* seeds or its litter were incorporated to previously amended tailings substrates. Our experiment showed that the effect of plant and litter on microbial composition was dependent on the previous presence of amendments, especially compost. At those treatments containing compost, the higher growth of plants was able to shape microbial composition (both, bacterial and fungi), while at the non-amended and biochar-only containing treatments (where a scarce growth of plants occurred), litter showed a more important role, especially in shaping bacterial composition. The higher growth of plants favoured some bacterial groups, whose relative abundances were previously depleted in the compost containing treatments (e.g. *Actinomycetales*) and other groups related to plant rhizospheres (e.g. *Rhizobiales*). These results revealed the ability of plants for shaping their own rhizospheric microbiome when the amendments contribute to ameliorate the low fertility of mine tailing soils. The employment of the combinations biochar-compost could result optimal to achieve the establishment of a vegetation cover at tailings, providing at the same time an effective tool to stimulate plant growth (higher plant biomass), a recalcitrant source of organic matter (biochar) and a support for the development of a plant rhizospheric microbiome.

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CHAPTER 1. Introduction

1.1. Soils polluted by mining activities: mine tailings

Industrial activities such as mining and processing of metal ores are important causes of environmental degradation worldwide (Dudka and Adriano, 1997). Among the impacts of mining, the generation of large quantity of waste materials is considered the most critical factor for environmental health. Several reports (Dudka and Adriano, 1997; Rico et al., 2008) specifically consider mine tailings to be the major source of pollution in former mining areas due to the high risk of spread of toxic elements such as As, Cd, Cu, Pb, Sb, Zn, etc., which can lead to a multi-elemental contamination of the environment (Dudka and Adriano, 1997).

Mine tailings are mixtures of crushed rock and processing fluids from mills, washeries or concentrators (Figure 1.1) that remain after the procurement process of ore particles from the matrix of less valuable rock (Kossoff et al., 2014). These processes can use physical and chemical methods, being the last ones those of most damaging consequences to the environment (Hoskin and Ireland, 2000). The resulting wastes in form of sludge are usually deposited in storages behind dammed impoundments, which are often called as ‘tailings ponds’ or ‘tailings dams’ (Kossoff et al., 2014). These sludges are stored under water to prevent the formation of surfaces particles and acid mine drainage. When the dumping activity is over, the water is evaporated or drained and the mine waste formed a big pile of tailings. Two major concerns are related to the environmental management of tailings: the risk of collapse and the spread of metal(loid) particles from their surfaces.



Figure 1.1: Example of mine tailings located in Cartagena-La Unión Mine District in Southeast Spain.

Structural instability of mine tailings may cause their collapse under unfavourable rainfall or seismic events. Collapses of mine tailings have been reported worldwide, being Europe the second zone with the highest number of events, only exceeded by USA (Rico et al., 2008). In Europe, most of the collapsing events that occurred in operational active tailings were due to extreme rainfall events (Rico et al., 2008). The most relevant mine tailings spill in Spain, both from quantitative and qualitative point of view, took place during spring in 1998 at the surroundings of Doñana National Park (Aznalcollar) in the South of Spain (Grimalt et al., 1999, Figure 1.2). In this event, high quantities of toxic acidic waters and mud containing high metal(loid) concentrations were released into the Agrio River and then passed into the Guadiamar River (Grimalt et al., 1999; Garrido, 2008). In total, 4,286 ha of land surface was affected by the toxic mud, of which 2,557 ha were devoted to agriculture activities. In addition, 4.2 and 0.19 % of total surface of Doñana Natural and National Parks, respectively, were damaged by tailings wastes. In relation to fauna, most of fish and shellfish in the water courses died due to the polluted overflow and the change of water parameters. However, the cause of death of several terrestrial vertebrates, which were found in this area, remains still undetermined (Grimalt et al., 1999).



Figure 1.2: Photograph of the Aznalcollar's accident from Morillo (2018).

At the Cartagena-La Unión Mining District in Southeast Spain (area covered in this PhD Thesis) some collapsing events at tailings have been also registered. The most important collapse took place at the “Brunita” tailings pile in 1972 (Figure 1.3) as a result of a strong rainfall event,

which caused the flooding and burial of most of the La Unión cemetery, and the death of one person (García-García et al., 2004).



Figure 1.3: Photograph of the Brunita tailings pile near La Unión.

Under favourable climatic/seismic conditions, and thus not likely collapsing situations, the spread of contamination from tailings surfaces becomes the main risk to preserve the environmental health. Tailings are usually characterized by poor soil quality parameters such as low pH, high heavy metal(loid) concentrations, lack of nutrients (low fertility), high electrical conductivity and lack of soil structure. These edaphic conditions hinder the establishment and growth of plants on tailings surfaces, which remain bare and thus, exposed to wind and water erosion (Conesa and Schulin, 2010). In addition, if erosion processes intensify during long term periods, the risk of collapsing could also rise up. Brotons et al. (2010) concluded that wind vector is of special environmental health concern at tailings located in arid climate areas and containing small size particles (silt and clay). In these conditions, agricultural activities and urban populations in tailings' vicinities could be seriously impacted by wind transported particles. Water erosion can carried out large quantities of tailings materials through huge distances specially, under strong rainfall events, as it occurs in Mediterranean climate mining areas such as the Cartagena-La Unión Mining District (Conesa and Schulin, 2010). In addition to the suspended transported particles, flowing water from tailings may contain dissolved metals, particularly under acid pHs, which may contaminate ground and surface waters and expose aquatic fauna to

toxicological effects (Sanchiz et al., 2000; Conesa and Schulin, 2010). All the pathways that spread particles from tailings increase the risk of metal uptake into flora and fauna causing a serious case of metals accumulation in food chain (Conesa and Schulin, 2010, Figure 1.4).

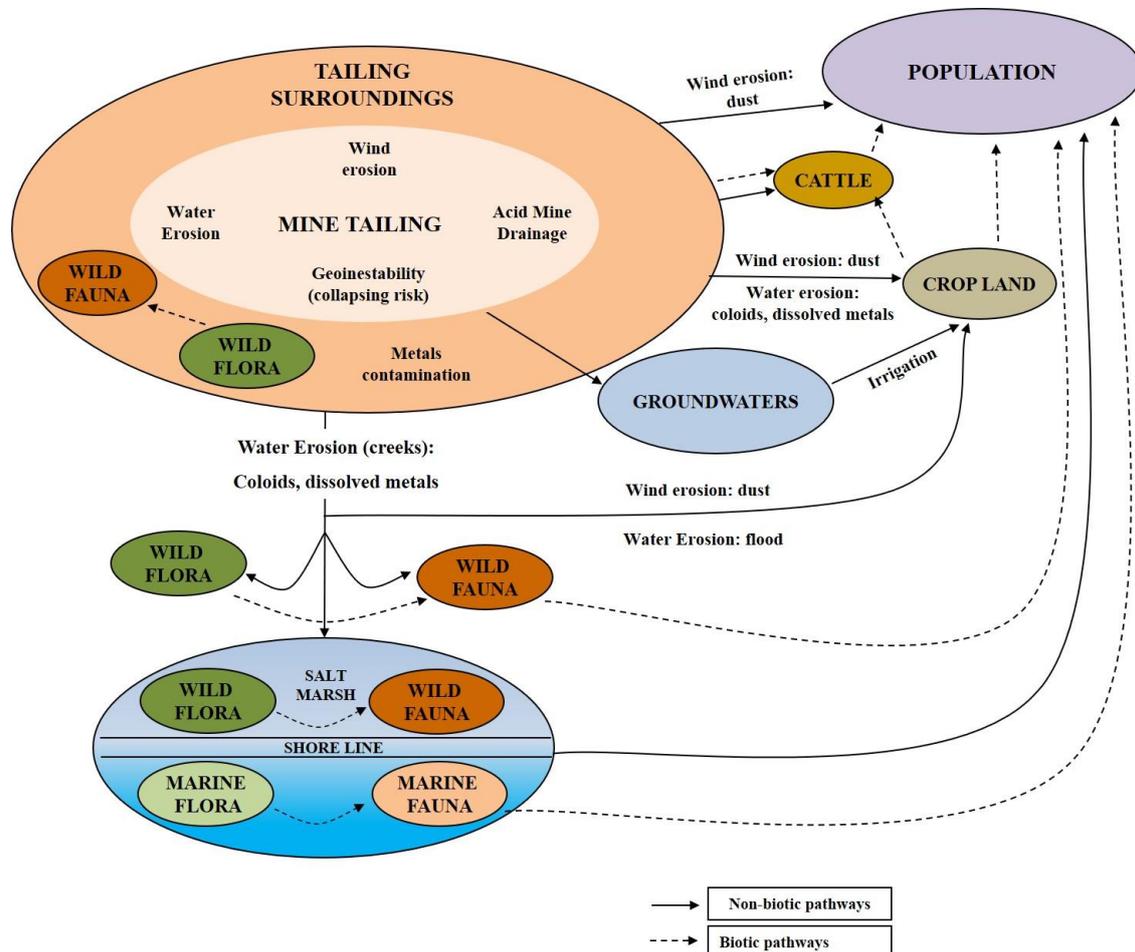


Figure 1.4: Metal transmission routes from mine tailings to biota. Continuous lines are non-biotic pathways. Dashed lines are biotic pathways. Adapted from Conesa and Schulin (2010).

1.2. Restoration techniques for mine tailings: the specific case of phytomanagement by phytostabilization

The aforementioned environmental risks posed by mine tailings have shown the need of developing feasible restoration techniques for their stabilization. Conventional engineering techniques based on physical methods are very expensive and only justified under critical environmental health situations (e.g. near urban areas or high risk of collapse). Physical methods consist of covering tailings surfaces with layers of different types of solid materials, e.g.

innocuous remains belonging to waste rocks from stripping operations or topsoils from non-polluted sites (Mendez and Maier, 2008a). In some cases, the most convenient option for erasing completely the environmental risk is the removal of tailings and their later dump into landfills. Chemical techniques involve the addition of chemical agents such as lignin sulphonate or resinous adhesives, which generate crusts on the tailings surface, immobilizing *in situ* the toxic particles and protecting tailings surfaces from the water and wind erosion (Tordoff et al., 2000; Mendez and Maier, 2008a). However, chemical techniques may present several limitations such as their usually short term lasting effects, which implies the need of continuous monitoring (Tordoff et al., 2000). For instance, lime amendments decrease their immobilizing efficiency with the time especially under acidic conditions, requiring new applications (Ruttens et al., 2010).

The need to achieve a long-term stabilization of tailings has resulted in the development of remediation techniques based on the use of plants. These methods, included under the term of phytotechnologies, are currently gaining scientific and technical attention due to their benefits in terms of cost-effectiveness and reliability for long term periods (Mendez and Maier, 2008a). In addition, they allow to enhance aesthetic landscape attributes (Mendez and Maier, 2008b). There are several types of phytotechnology techniques depending on the established environmental goal (Figure 1.5), being two ones the most widely employed: phytoextraction that focuses on the accumulation of metals on shoot tissues, which have to be later removed and managed; and phytostabilization, whose aim is the immobilization of metals within plants rhizospheres (Mendez and Maier, 2008b). Other phytotechnology techniques include rhizofiltration, where roots are used to retain contaminants from polluted waters (Wong, 2003); phytovolatilization, which promotes the volatilization of contaminants or phytodegradation, which favours their transformation into less harmful compounds (Wenzel, 2009).

Phytoextraction Phytoaccumulation	Metals are accumulated in plant tissues	<ul style="list-style-type: none"> - Bioaugmentation-assisted phytoextraction (Combined with mycorrhiza) - Phytomining (Economic profit of metal accumulated by plants) - Chelated-assisted phytoextraction (Adding chelants to soil combined with plants) - Phytoextraction (Metals are recovered from leaves excretions)
Phytofiltration		<ul style="list-style-type: none"> - Biosorption (Metals are absorbed or bound in living or not living plant material) - Rhizofiltration (Metals are absorbed or bound in roots) - Blastofiltration (Metals are absorbed or bound in seedlings)
Phytodegradation Phytotransformation	Organic pollutants are degraded by plants through plant uptake (and metabolic processes) or through the release of compounds in rhizosphere (without the intervention of microorganisms)	
Phytovolatilization	Contaminants are taken up by plants and volatilized to the atmosphere through leaves	
Phytoimmobilization	Metals are taken up into leaves which “store” them after falling onto soil	
Phytostabilization	Immobilization of the contaminant (metals) in soil by means of absorption/accumulation/ adsorption onto roots or precipitation within the rhizosphere	<ul style="list-style-type: none"> - Phytoexclusion (Use of plants with low metal uptake) - Aided phytostabilization (Improvement by using amendments) - Hydraulic control (Decrease pollutants in leaching) - Phytorestoration (Phytostabilization employing native plant species)
Plant-assisted bioremediation Rhizosphere bioremediation Rhizodegradation Rhizoremediation Enhanced rhizosphere degradation Plant-assisted degradation Plant-aided in situ biodegradation	Organic pollutants degraded in rhizosphere with the intervention of microorganisms	

Figure 1.5: Current classification of most frequently used phytotechnologies for soil remediation. Adapted from Conesa et al. (2012).

Recent works on phytotechnologies consider more adequate to name these techniques with the term phytomanagement. This concept was introduced by Robinson et al. (2009) and refers to the *engineering or manipulation of soil-plant systems to control the fluxes of trace elements in the environment*. This approach considers that the aforementioned categories of phytotechnologies do not work as “airlocks” but as main “mechanisms” among different biogeochemical and ecological processes. For instance, while hyperaccumulator plant species are required for phytoextraction, the selected plants for phytostabilization should not accumulate high concentration of metals in their shoot tissues (Wood et al., 1995; National Research Council, 2005; Mendez and Maier, 2008b; Figure 1.6). However, it is reasonable to admit that plants employed in phytostabilization may also uptake a certain amount of pollutants into shoots and those ones used in phytoextraction may fix some contaminants within their rhizospheres. Therefore, the goal should be the management of the whole fluxes of metals in the soil-plant system, including its related ecological successional processes, and not focusing on a single part of that system.

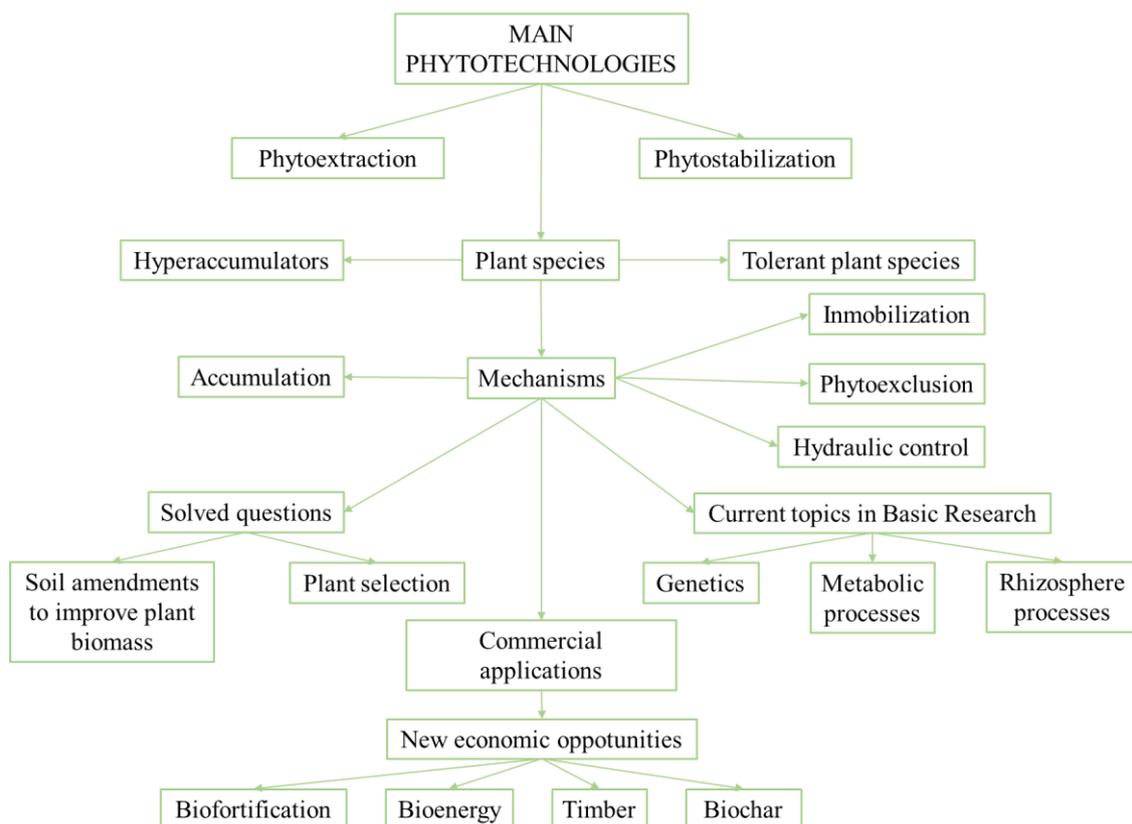


Figure 1.6: Phytostabilization and phytoextraction application. Current and future development. Adapted from Conesa et al. (2012).

For the specific case of mine tailings, phytomanagement by phytostabilization has been considered as a promising tool to effect their surface stabilization (Mendez and Maier, 2008a; Párraga-Aguado et al., 2013). This method is focused on the generation of a stable vegetation cover employing metal(loid) tolerant plant species with robust root systems, where metals could be immobilized. The immobilization of metal(loid)s within rhizospheres might be achieved by the occurrence of several processes such as adsorption on root surfaces, accumulation into root tissues, complexation with root exudates and/or precipitation in inorganic or organic compounds onto root surfaces (Wong, 2003; Wenzel, 2009). In addition, the establishment of plants fixes soil particles through their attachment by roots, which may prevent tailings surfaces from wind and water erosion, decreasing their environmental risks. But the beneficial effect of plants may last longer through triggering successional ecological processes, which might facilitate the self-sustainment of the vegetation cover at long term.

According to Tordoff et al. (2000), there are several critical factors related to plant selection to be taken into account in the phytostabilization of metal(loid) enriched tailings: a) abiotic factors (climate, tailings characteristics), b) plants tolerance to drought and salinity, especially in arid and semiarid areas, c) metal(loid) tolerance of selected plant species and d) main plant mechanism for detoxification of metal(loid)s (rhizosphere fixation or translocation into the aerial tissues). Plant species employed in the phytostabilization in semiarid areas must deal with several co-limiting edaphic factors: acid pHs, high electrical conductivity, high metal(loid) concentrations, low nutrient concentrations or low water holding capacity. In order to overcome these issues, research should focus on several issues: i) effecting a suitable selection of tolerant plant species to co-limiting edaphic factors ii) the assessment of soil amendments to ameliorate soil limitations and facilitate/improve plant establishment; and iii) the role of soil microbiology as a critical factor to facilitate plant establishment by improving its adaptation to harsh soil conditions.

1.2.1. Plant selection: the role of pioneer vegetation at mine tailings restoration

Nowadays, the selection of plant species for the phytomanagement by phytostabilization in mine tailings is focused on searching within native plants from the local sites surrounding tailings (Mendez and Maier, 2008a; Párraga-Aguado et al., 2014b). There are several reasons that justify this approach: to avoid the introduction of potentially invasive species and assure the adaptation of the selected plant species to the local climate conditions. Traditionally, studies on phytoremediation have been focused on selecting the most tolerant plant species to contaminants, regardless their ecological interactions in field. In this way, weed species such as *Prosopis juliflora* (Usman et al., 2019) or *Zygophyllum fabago* (Boojar and Tavakkoli, 2011) have been proposed as suitable candidates for phytoextraction/phytostabilization of metal polluted soils obviating their noxious role and high ecological competitiveness. This may cause ecological disturbances in those areas where are introduced. Current research is moving towards a more integrative approach of the selected plant species, including ecological interactions as a factor to

be critically considered in the phytomanagement projects. For this reason, candidate species are desirable to come from local native plant species. In this way, the adaptation to specific climate conditions is assured (especially important in arid and semiarid areas) along with the absence of later disturbances in their ecological interactions (Conesa et al., 2006). The search of the most adequate plant species benefits from field surveys in ancient non-restored tailings, where adapted species can grow due to the low number of competitors. The extreme edaphic conditions at tailings act as a filter, selecting those species that can cope with soil fertility co-limitations: heavy metals, drought, high salt concentrations, low levels of nutrients, etc (Párraga-Aguado et al., 2013). These plant species that initially colonize tailings surfaces are known as pioneer vegetation and are considered as a key factor to promote the establishment of late successional plant species in tailings (Párraga-Aguado et al., 2013, 2014b).

Pioneer plants initially colonize barren tailing surfaces at specific edaphic niches, where properties are more favourable for plant establishment (Párraga-Aguado et al., 2013). By their later growth, pioneer vegetation improves soil organic matter and the activity of plant promoting microbiology (Wenzel, 2009). This sets the basis for edaphic and ecological successional processes, which in turn will improve soil fertility and will allow the establishment of other plant species, which did not tolerate the former harsh soil conditions. At long term, this process of facilitation may lead to the formation of vegetated patches at tailings composed by several plant species, including late successional species, which are known as fertility islands (Figure 1.7). Fertility islands require the assembling of several types of plant species with complementary ecological functionality (Párraga-Aguado et al., 2014b). For instance, the growth of grasses roots is restricted to first soil centimetres, while shrubs or trees may explore deeper horizons. In this way, they do not compete for water or nutrient resources. The key for the long term stability of the fertility islands is the presence of trees: the “canopy” effect of trees prevents erosion and decreases water evaporation (shadow), the strong root systems of the trees allows to stabilize higher volumes of soil, etc.

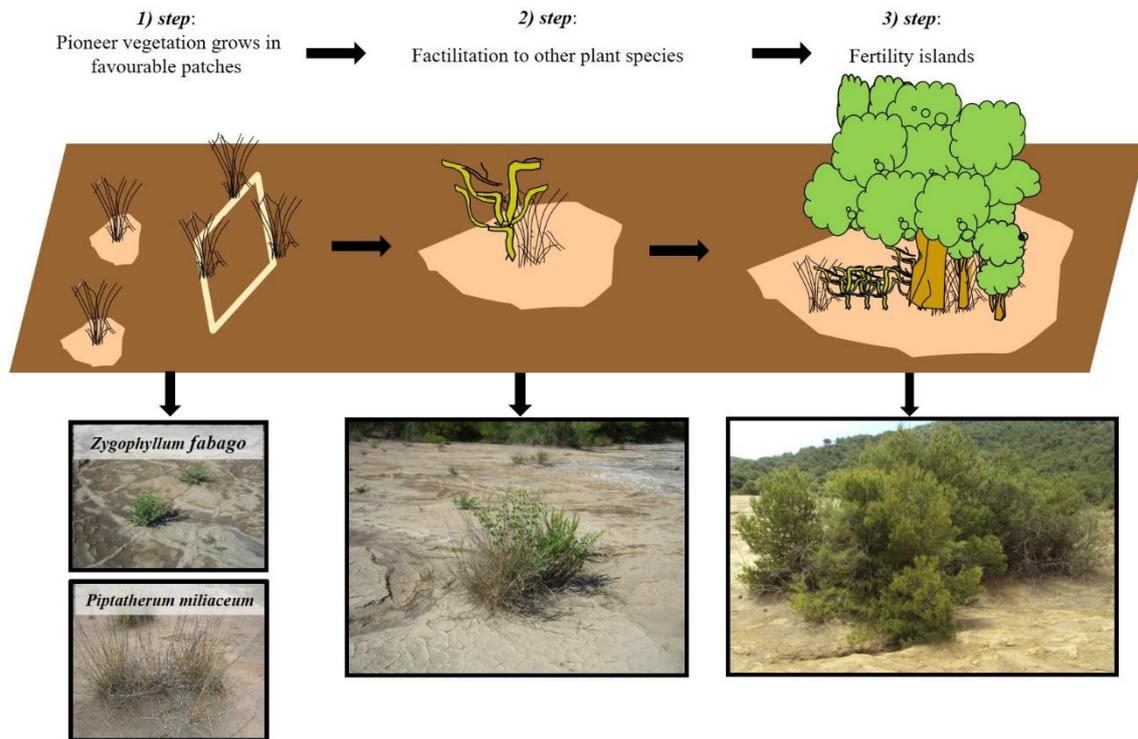


Figure 1.7: Schematic representation of the process from pioneer vegetation to fertility islands.

Pioneer vegetation usually consists of annual herbs/grasses in many cases of halophytic characteristics. For instance, the Gramineae *Piptatherum miliaceum*, the Zygophyllaceae *Zygothymum fabago* or the Chenopodiaceae *Salsola kali* have been described as pioneer colonizers in semiarid mine tailings areas of Southeast Spain (Párraga-Aguado et al., 2013, 2014a,). On the other hand, and under the same climatic conditions, tree species as *Pinus halepensis* or *Tetraclinis articulata* have been considered as key species to form fertility islands (Párraga-Aguado et al., 2014b). More specific information on these type of plant species will be discussed in the Chapter 2 together previous results in the studied area.

1.2.2. Soil treatments through amendments (compost and biochar)

The use of soil amendments has become a key factor to facilitate plants growth during their early phase of establishment at tailings (Gil-Loaiza et al., 2016; Forján et al., 2017). Amendments might ameliorate soil constraints at tailings by promoting, among others, adequate pHs, immobilization of metal(loid)s, suitable nutrient contents and water retention capacity or a feasible soil microbial composition (e.g. González-Alcaraz et al., 2011; Clemente et al., 2012;

Zornoza et al., 2013b). In a general point of view, two main types of soil amendments can be distinguished: inorganic and organic.

Inorganic amendments can include chemical fertilizers for providing readily available nutrients for plants (Conesa et al., 2007b; Cordell et al., 2009) or soil conditioners with the aim of modifying specific soil properties such as pH (Kumpiene et al., 2008). For instance, lime amendments have been applied to rise pH in acidic tailings substrates (Conesa et al., 2007a; González-Alcaraz et al., 2011), amendments containing phosphate rocks have been used to immobilize Pb (Theodoratos et al., 2002) or nutrient solutions have been included in periodical irrigations for supporting plant growth (Conesa et al., 2007b). Recent research on inorganic amendments include the use of nanomaterials such as nanoparticles of iron sulphide, oxides or phosphate (Liu and Lal, 2012). The advantages of these kind of amendments lay on the fast response in modifying soil properties or improving plant growth. However, these beneficial effects may last shortly and in most of cases new applications are needed. In fact, acid neutralization requires a specific assessment to assure the long-term pH stability and make possible a suitable plant growth (Gurung et al., 2018). Under strongly acidic conditions neutralizing materials might be gradually “consumed” by the existing sulphides, which may lead to a remobilization of metals (Sarkar et al., 2018). In addition, the application of phosphate rocks as a method for immobilizing Pb could bring some drawbacks in those tailings containing high As and Sb concentrations because of favouring their mobilization (Theodoratos et al., 2002; Kilgour et al., 2008). Nitrate containing amendments, although have shown a suitable effect in favouring plant growth, may alter ecological interactions when applied in field conditions by favouring those opportunists or nitrophilous plant species over late successional ones (of less competitive character) (Conesa et al., 2007b). For this reason, a specific assessment is required before inorganic amendments be applied in the field.

Organic amendments are widely considered as the most suitable option for improving plant growth in metal(loid) enriched tailings (Clemente et al., 2005; Párraga-Aguado et al., 2017b; Visconti et al., 2020). The specific effects of the organic amendments on soil properties depend

on their nature, the rate of application and the characteristics of the targeted tailings. A general view on the benefits of organic amendments at tailings includes the improvement of physical, chemical and biological soil properties. In relation to physical properties, organic materials may trigger an incipient development of soil structure by the formation of soil aggregates, which in turn increase water soil retention capacity (Sarkar et al., 2018). From the chemical point of view, organic amendments fill up some of those lacking nutrients at tailings, which are essential for plant growth, such as nitrogen (Martínez-Oró et al., 2019). Moreover, organic matter also increases cation exchange capacity and chelating properties favouring the temporary immobilization of metal(loid)s (Bolan et al., 2014). Finally, from the biological point of view, there is a critical link with the support of soil microbiological activity and in turn the sustain of nutrient cycles (e.g. C, N) (Pepper et al., 2012; Valentín-Vargas et al., 2014). Although there is a general assumption of the benefits of the organic amendments, recent research have revealed the need of specific assessments in order to avoid undesirable long-term effects including negative disturbances in plant and microbial ecological relationships (Huang et al., (2012) and Martínez-Oró et al., (2019), respectively), losses of N to atmosphere (Chen et al., 2010), excessive increase of soluble salts (Párraga-Aguado et al., 2017a) or the increase in the available pools of certain metal(loid)s (Schwab et al., 2007; Cuske et al., 2016).

There are many types of organic materials employed in tailings: from fresh litter materials used as mulching on the surface to mature composts which are mixed within the first tailings centimetres (Tripathi et al., 2016). In the specific case of tailings, it is interesting to link the application of amendments with by-products generated by other activities, favouring circular economy (Párraga-Aguado et al., 2017a, 2017b). These by-products may include sewage sludge from urban wastewater plants, soil urban wastes, animal manures, wastes from agro-food activities, etc (e.g. Kumpiene et al., 2008; Clemente et al., 2015, 2020). Organic amendments coming from farming activities are particularly appreciated because of the adequate composition in nutrients, especially N, P and K (Epstein, 2011; Zornoza et al., 2013a). Due to the high metal(loid) concentrations of tailings, several authors consider reasonable the application of by-

products with certain metal(loid) load such as those ones coming from urban treatment plants (Párraga-Aguado et al., 2017a).

Recent research has promoted the employment of biochar, coming from the pyrolysis of organic materials, which contain higher proportion of stable recalcitrant carbon over other forms of labile carbon (Sohi et al., 2010; Hammond et al., 2011). Benefits of biochar include the increase of soil water and nutrients storage and the stimulation of microbial activity (Lehmann and Joseph, 2009). For these reasons, biochar amendments are being widely employed in the reclamation of metal(loid) polluted soils (e.g. Beesley et al., 2010; Fellet et al., 2011; Zhang et al., 2013; Puga et al., 2015; Jones et al., 2016; Álvarez-Rogel et al., 2018).

Several researchers have shown that the use of combined amendments with contrasting properties generates more positive results than when used separate (Rodríguez-Vila et al., 2014; Oldfield et al., 2018). Combining organic amendments like compost manure with biochar may provide readily available nutrients (coming from manure), which stimulate microbial activity (Wu et al., 2016) and a stable carbon source with higher potential for retain nutrients and water (coming from biochar) (Oldfield et al., 2018).

1.2.3. Importance of native microbiology for soil stability

Nowadays, soil microbiology is considered a key factor to evaluate the environmental health of ecosystems. The beneficial features of soil microorganisms' activity include the recycling of nutrients such as nitrogen or carbon, the stimulation of plant growth (directly or indirectly), the improvement of soil structure and water-holding capacity, or their participation in the remediation processes through the degradation or fixation of pollutants (Coyne, 2000; Tugel et al., 2000).

The soil microbial community is formed by a high diversity of microorganisms, being the main groups: bacteria, archaea, fungi, protozoa and algae (Coyne, 2000; Pepper et al., 2015). Bacteria and archaea are prokaryote organisms characterized by the lack of a true nucleus and

membrane-bound cell organelles, such as mitochondria or chloroplasts. The archaea domain is mainly composed by extremophiles microorganisms and little information about its presence in non-extreme environments is available (Pepper et al., 2015). The bacteria domain is composed by a high diversity of microorganisms, which are involved in a great variety of soil biogeochemical processes and live in a wide range of environmental conditions (Coyne, 2000; Pepper et al., 2015). On the other hand, the fungi, protozoa and algae domains are defined as eukaryotes. They contain a true nucleus and membrane-bound cell organelles (Pepper et al., 2015). While bacteria are considered the most numerous microorganism group in soils (Coyne, 2000), fungi are known to have the greatest biomass (Pepper et al., 2015). Although the main function of fungi is the degradation of organic matter, this domain can also take part in a wide variety of soil processes due to its high lifestyle diversity (e.g. mycorrhizal or pathogens) (Coyne, 2000; Pepper et al., 2015). Protozoa are mainly heterotrophic and feed directly by preying other organisms (bacteria, fungi and algae). This may effect indirectly the structure and function of the edaphic microbial community involved in the degradation of organic matter (Coyne, 2000; Pepper et al., 2015). Finally, the algae domain is formed by eukaryotic aerobic photosynthetic microorganisms that have showed a high dependence of water, and that may colonize climate extreme ecosystems (Coyne, 2000; Pepper et al., 2015). Considering the features of the aforementioned microorganism groups, bacteria and fungi seem to play a more important role in the most general edaphic processes and soil biogeochemistry cycles, including a critical relationship with plant growth. Thus, this PhD Thesis will we focused in these two domains (bacterial and fungi).

Both bacteria and fungi are known to be the most abundant microorganisms in most of soils, even so, it is difficult to estimate their actual diversity, since for example, in the case of bacteria, only 1% of species can be cultivated (Coyne, 2000). However, current techniques of molecular biology, based on 16S rRNA (16S ribosomal RNA) and ITS (nuclear ribosomal Internal Transcribed Spacer region), for bacteria and fungi, respectively, allow to calculate relative abundances for different taxonomic levels (phylum, class, order, etc). Additionally, it is

possible to calculate their evolutive distances by generating a phylogenetic tree (Toju et al., 2012; Thijs et al., 2017). Therefore, this methodology can provide a reasonable view of bacterial and fungal composition in soil samples (Atlas and Bartha, 2002; Schloss and Westcott, 2011).

Bacteria include a high variety of organisms. Their classification is based on several criteria (Figure 1.8). For instance, according to the type of carbon source that they employ bacteria can be classified into autotrophic or heterotrophic. The first group refers to those bacteria that obtains carbon from an inorganic carbon source (usually CO₂), while the latter are those ones that obtains carbon from organic matter compounds. In turn, bacteria which use organic matter are usually saprophytes, also called decomposers (e.g. *Actinomycetes*) (Tian et al., 2014). This group degrades organic compounds such as those contained in plant litter and plays an important role in providing nutrients for soil other biota (Tugel et al., 2000).

Another classification is based on the type of the reducing agent (organic or inorganic) used to obtain energy and classifies bacteria into organotrophs, which use organic compounds (e.g. order *Pseudomonadales*) or lithotrophs (e.g. order *Chromatiales*), which obtain energy from inorganic compounds of nitrogen, sulphur, iron or hydrogen (Tugel et al., 2000; Kersters et al., 2006; Pepper et al., 2015;). Finally, there is an important environmental feature of several bacterial groups coming from the relationships that can be established with other organisms, especially with plants. Thus, depending on that type of interaction, bacteria can be classified as mutualist or pathogens. The mutualist lifestyle, so-called symbiotic, is based on an obligatory relationship between two organisms, from which both get profits. One widely known symbiotic interaction is that established between nitrogen-fixing bacteria, such as *Rhizobium* genera, and legume plants (Tugel et al., 2000; Atlas and Bartha, 2002). On the other hand, those pathogenic groups (e.g. some species of *Pseudomonadales* order) can cause different diseases in the plants including morphological or metabolic alterations (Tugel et al., 2000; Atlas and Bartha, 2002).

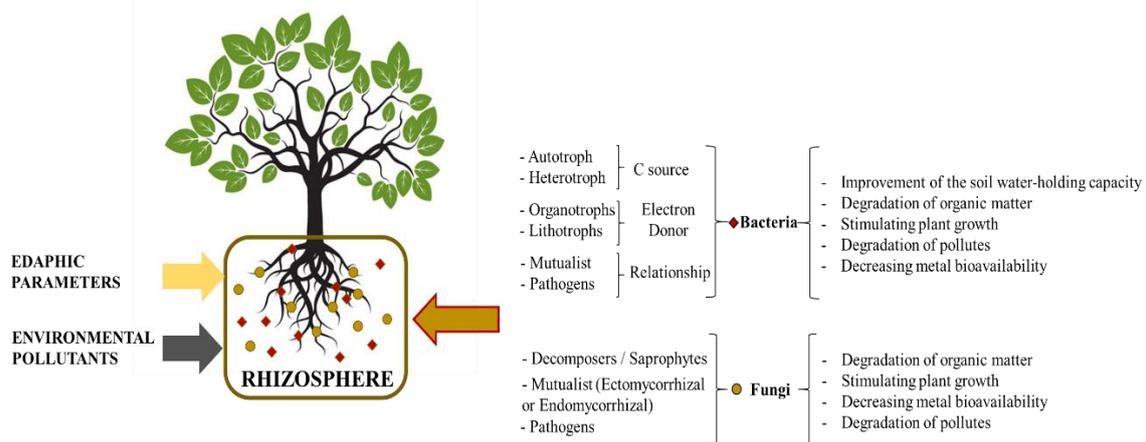


Figure 1.8: General classification of bacteria and fungi and their main soil functions.

Within the bacterial domain, there are a high diversity of bacteria phyla, which are implicated on soil biogeochemistry cycles. Some of the most important taxa, especially by their adaptability to wide ranges of environmental conditions, are the *Proteobacteria*, *Actinobacteria* and *Acidobacteria* phyla (Roesch et al., 2007). The *Proteobacteria* phylum is the largest bacterial group with high variety of lifestyle and plays important roles in critical soil nutrient dynamics such as the nitrogen fixation (e.g. *Rhizobiales* order), organic matter decay (e.g. *Burkholderiales* order) or the sulphur cycle (e.g. *Chromatiales* order) (Kerstens et al., 2006). The *Actinobacteria* phylum is another abundant bacterial group due to its adaptative morphology, which shows intermediate characteristics between fungi and bacteria, and a remarkable capacity for degrading organic matter (Alvarez et al., 2017). Taxa belonging to the *Actinobacteria* phylum, and especially those included in the *Actinomycetes* order, are known to participate in the degradation of recalcitrant organic matter (Tian et al., 2014). In relation to the *Acidobacteria* phylum, although it is not so well studied as the aforementioned groups, it has shown a high representation in a great variety of soils (Jones et al., 2009). In addition to these phyla, other less abundant bacterial phyla such as *Bacteroidetes* or *Planctomycetes* have also shown an important role in soil processes such as decomposition of organic matter (Fierer et al., 2007; Nie et al., 2015).

In the case of the fungal domain, taxa can be clustered in three functional groups according to the type of energy source: decomposers, mutualists or pathogens (Tugel et al., 2000; Webster and Weber, 2007, Figure 1.8). Decomposer fungi, also called saprophytes, are

heterotrophic microorganisms that can transform complex organic substrates as lignin or cellulose into carbon dioxide or simpler organic molecules. The latter are usually organic acids that are accumulated as humic acids enriched organic matter, contributing to the storage of nutrients in the soil (Tugel et al., 2000; Webster and Weber, 2007). Mutualistic fungi colonize plant roots and are known as mycorrhizal fungi. In this interaction, both plants and fungi can obtain different profits: fungi mainly get carbon from plants whereas plants can access to soil nutrients (phosphorus, nitrogen, etc.). This functional group can be classified into ectomycorrhizal fungi such as the *Thelephorales* order (they grow mainly on tree root surfaces) and endomycorrhizal fungi such as the *Glomales* order (they grow inside root cells) (Tugel et al., 2000; Webster and Weber, 2007). Within endomycorrhizal fungi, arbuscular mycorrhizal fungi is a special group which develop vesicles or arbuscules in the root cells (Webster and Weber, 2007). Pathogenic fungi can cause diseases or metabolic alterations in plants (e.g. effect of *Fusarium* on tomato plants) (Atlas and Bartha, 2002).

In the case of fungal domain, there are two dominant phyla in soils, *Ascomycota* and *Basidiomycota*, which are known to play an important role in soil biogeochemical processes, including their participation in N (Webster and Weber, 2007) and C (Lundell et al., 2010; Ma et al., 2013) cycles. The *Ascomycota* phylum is the largest group of fungi, and includes a high variety of lifestyles, from saprotrophs, necrotrophic or biotrophic parasites to endophytes or endomycorrhizal fungi (Webster and Weber, 2007). On the other hand, the *Basidiomycota* phylum is mainly composed by saprotrophic and ectomycorrhizal fungi (Webster and Weber, 2007).

Due to the multiple functions of microorganisms in ecosystems, recent approaches include soil microbiology as a reliable soil quality indicator for evaluating environmental health (Mendez et al., 2007). Nowadays, this characteristic results critical due to the huge areas worldwide affected by degradation processes, including those polluted sites (Pepper et al., 2015). Alterations in soil properties in polluted soils (pH, salinity, organic matter concentration, concentration of pollutants, etc.) can lead to variations on composition or functionality of soil

microorganisms. For instance, Marcin et al. (2013), when analysing the microbial communities in soils affected by different levels of heavy metals, determined a strong dependence of bacterial diversity and community structure with pH (e.g. higher pH values favoured some bacteria groups such as *Deltaproteobacteria* or *Firmicutes*). On the other hand, Thiem et al. (2018), analysed the bacterial and fungal microbiome of black alder (*Alnus glutinosa* Gaertn.) rhizosphere at two forest test sites (saline and non-saline) in two different seasons (spring and fall) and showed that bacterial and fungal structure of alder root microsymbionts and endophytes depended mainly on four soil chemical parameters: salinity, phosphorus, pH and saturation percentage. In the case of fungi, Carrasco et al. (2006) determined that soil salinity and heavy metals concentrations specifically affected the degree of colonisation of plant roots by arbuscular mycorrhizal fungi in tailings polluted soils.

However, some microorganisms have shown tolerance or resistance to extreme conditions and to the presence of pollutants (Pepper et al., 2015). This feature of microorganisms can be used for environmental restoration purposes. In relation to the specific case of mine tailings phytomanagement, several authors highlighted the importance of soil microbiology in the establishment of self-sustainable vegetation, especially in arid and semiarid climates, where the limiting factors for plant growth include salinity and/or drought (Mendez and Maier, 2008b; Huang et al., 2012). Metal impacted ecosystems, such as those found in mining areas, develop specific microbial communities, which not only are able to exhibit tolerance or resistance to contaminants but also may sustain biogeochemical processes under harsh environmental conditions not tolerated by most of higher organisms (Huang et al., 2011; Fernandes et al., 2018;). For instance, some endophytic bacteria can act as plant growth promoting bacteria providing specific tolerance to heavy metals as Cd, Pb or Zn (Ma et al., 2016). Some authors, such as Chen et al. (2013) have shown the positive effects of inoculating this type of bacteria (e.g. *Pseudomonadales* and *Burkholderiales*) on the performance of plants growing at polluted soils. Similar to those results, the inoculation of the fungal *Glomerales* order improved the establishment of vegetation in metal polluted soils (Abbaslou et al., 2018). Thus, these results

make soil microbiology a critical factor to be taken into account in the restoration of mining impacted soils for two main reasons: 1) microbes ability to interact with plants rhizospheres, acting as filters to deplete metals phytotoxicity and, 2) microbes as supporters of C-N cycles providing available nutrients for plant growth (Thavamani et al., 2017).

1.3. References

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CHAPTER 2. Study area

The former Mining District of Cartagena-La Unión, whose activity is currently discontinued, has been considered one of the most important historical mining areas in the Iberian Peninsula. This long period of mining activity (first mines took place during Phoenicians) has caused a strong impact, not only in the local environment but also in the regional idiosyncrasy and social organization. In this section, general aspects of this mining area, focusing mostly on the environmental issues, are described.

2.1. Location, climate, flora and hydrology

The former Mining District of Cartagena-La Unión covers approximately 50 km² on the southeast of the Iberian Peninsula (37° 37' N, 0° 49' W– 37° 35'N, 0° 50' W) (Figure 2.1) and mostly occupies a territory that belongs to La Unión county (including La Unión and Portmán towns) and a small portion of the Cartagena county (including El Llano del Beal and Estrecho de San Ginés towns).

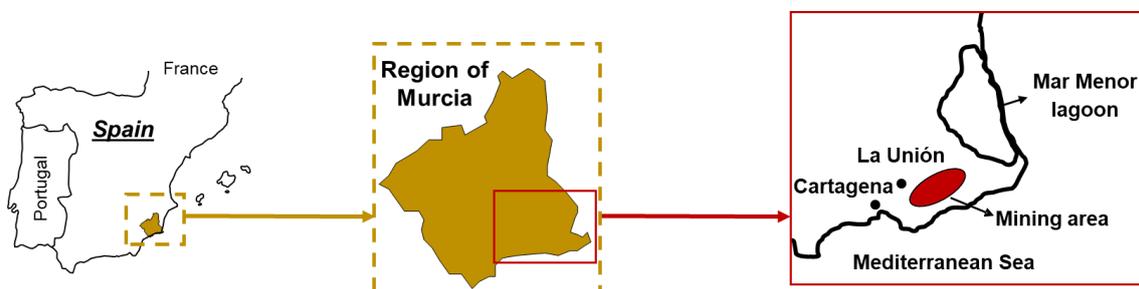


Figure 2.1: Location of the Mining District.

The mountain range of Cartagena, also known as Sierra Minera, crosses the District from west to east, parallel to the Mediterranean Sea. These mountains belong to the eastern part of the Betic Ranges just before they submerge into the Mediterranean Sea in the Cape of Palos.

The mountains of the Sierra Minera do not surpass 400 meters height. The topography on the southern face is steep, due to its proximity to the coast; in contrast, the northern face shows gentle slopes with long plains till the Mar Menor lagoon and belong to the landscape unit of the Cartagena Plain (Campo de Cartagena).

The climate of the zone is semiarid Mediterranean with annual rainfall of 250-300 mm, occurring mainly during spring and autumn, and an annual average temperature of 18 °C. Local winters do not show frosts, while summers are long and warm. Evapotranspiration rates can reach 800 mm year⁻¹, due to the high temperatures (especially during summers) and low precipitations. The location, near the coast, favours the occurrence of crypto-precipitations, which is critical for the sustainment of wild flora (Conesa, 2005; Jiménez-Cárceles et al., 2008).

The local hydrological net consists of ephemeral water courses or dry rivers, called *ramblas* (Figure 2.2). The water regime of these *ramblas* is related to seasonal rains, remaining dry most of the year. When occurring, water run-off can transport huge amounts of materials. There are seven main *ramblas*: five of them flow into the Mar Menor lagoon (Carrasquilla, Beal, Matildes, Miedo and Ponce) and the other two, to the Mediterranean Sea (El Gorguel and Portmán). The creeks which flow into the Mediterranean Sea are shorter (~ 3 km total length) and steeper (~ 10 %) than those ones flowing into the Mar Menor lagoon (7–12 km). The latter show an average slope of 3 %; their headwaters are steep and short (10 % slope), while their mouths are flat (< 1 % slope) and long. The mouths of the *ramblas* that flow into the Mar Menor are occupied by salt marshes and wetlands, where their sediments are deposited. The Mar Menor lagoon is a singular ecosystem in the entire Mediterranean basin. The lagoon and its related wetlands and salt marshes cover around 15 000 ha. Most of this area is protected by environmental protection laws and international conventions: Ramsar international site since 1994, it includes five Sites of Community Importance (SCI); it is a Site of Community Importance (SCI) to be incorporated into the Nature 2000 Network (EU Habitats Directive); it is also included as a Special Protected Area of Mediterranean Interest (SPAMI) (Barcelona Convention in 2001) (Conesa and Jiménez-Cárceles, 2007; Conesa and Schulin, 2010).

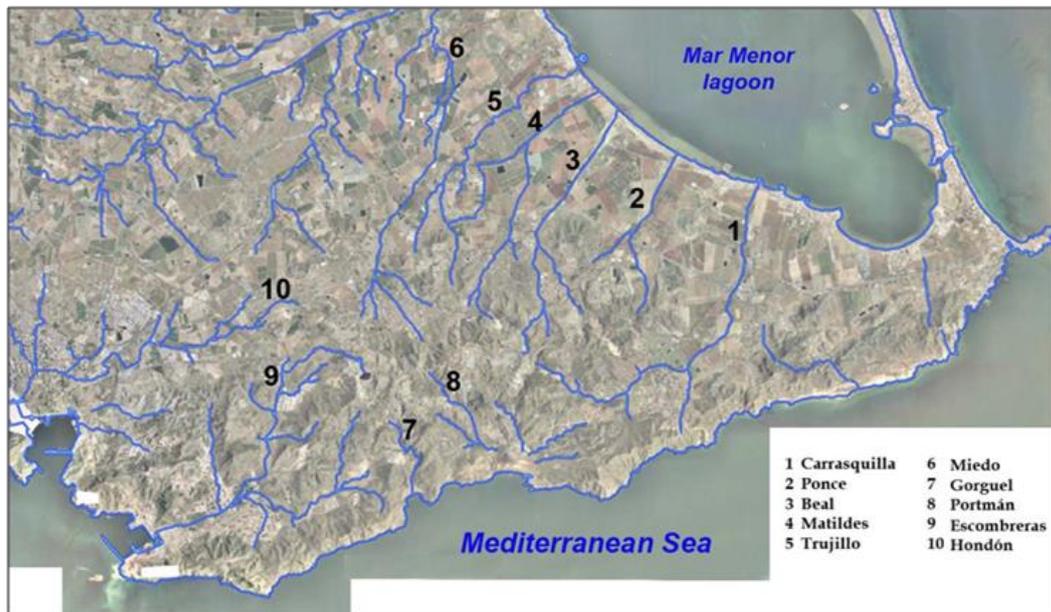


Figure 2.2: Hydrological net of the Mining District (Párraga-Aguado (2015) adapted from CHS (2016)).

The natural flora contains typical species of semiarid areas. The local forests are mainly composed by the tree *Pinus halepensis* and thickets of xerophytic shrubs (e.g. *Helichrysum decumbens*, *Lygeum spartum*, *Pistacia lentiscus*, *Rosmarinus officinalis*, *Stipa tenacissima*). Some species of the local flora are considered endemic and of high botanical interest (Martos-Miralles et al., 2001). Among the local plant species, it is highlighted the tree *Tetraclinis articulata* (Figure 2.3) which is considered as *a very rare species* according to the European Habitat Directive and has been included in the red list of threatened species (IUCN, 2012).



Figure 2.3: Image of a typical local open forest. At the bottom, *Chamaerops humilis* (palmito), at the centre of the image several individuals of *Tetraclinis articulata* (Ciprés de Cartagena).

Other local flora which is protected under regional environmental laws, include *Chamaerops humilis*, *Limonium carthaginense*, *Phyllirea angustifolia*, *Rhamnus alaternus*, and *Teucrium carthaginense* (BORM, 2003). Some of these endangered species, such as the *Limonium carthaginense* have a common habitat for growing the mining wastes that cover the area (Figure 2.4). The ecological relevance of this area is supported by the establishment of the Natural Park of “*Calblanque, Monte de las Cenizas y Peña del Águila*” (Site of Community Importance within the Nature 2000 Network).



Figure 2.4: Plant of *L. carthaginense* with flowers growing at a mine tailings pile. In the base of the *Limonium carthaginense* a *P. halepensis* seedling has appeared.

2.2. A brief history on mining activities in the area

The local history has been tightly associated to the mining activity, which already began more than two thousand years ago, during Iberians and continued with Phoenicians, Carthaginians and Romans. In this way, this area has one of the longest mining histories in the Iberian Peninsula. The local ore deposits had iron, lead and zinc as main metal components. Iron was contained in oxides, hydroxides, sulphides, sulphates, carbonates, and silicates; lead and zinc occur in galena,

sphalerite, carbonates, sulphates, and lead- or zinc-bearing (manganese, iron) oxides (Oen et al., 1975).

The local mines constituted an important source of metals for Romans, which established Cartago-Nova (current Cartagena) and settled there an important centre for managing the old roman Hispania. After Romans the mining activity suffered a strong decline during the Middle Age. The XIXth century is considered the starting point for the most intensive period of mining in the area. At first, with a mining activity through underground galleries (Figure 2.5) and traditional refining techniques based on gravimetric methods; and during the XXth century, with new extractive methods based on open cast mines (Figure 2.6) and the use of differential flotation as a more efficient technique for refining mining ores (Conesa et al., 2008b).



Figure 2.5: Underground gallery of the *Agrupa Vicenta* Mine (source: <http://www.parqueminerodelaunion.es/es/>).



Figure 2.6: Brunita open cast mine with its famous “red lake” (left) and the Gloria open cast mine (right).

The last period of mining activity corresponded to fifty years, from 1940 to 1990 and is considered as that with the most intensive activity in the local mining history. Several authors estimated that one third of the total mineral ore reserves of the area was mined in these fifty years (Manteca and Ovejero, 1992; Manteca and Berrocal, 1997). This quantity equals to the amount mined during the rest of history in the area. The mining activity was finally discontinued in 1991. Four causes are usually proposed for that end: first, the scarcity of mineral reserves; second, the low prices of metals in global markets; third, the reduction of public subventions for mining companies; and fourth, the growing awareness on environmental issues (Egea-Bruno, 2003) that was focused on the final closure of the dumping of mining wastes into the Portman Bay (Figure 2.7).



Figure 2.7: Demonstration of Greenpeace during the eighties to ask for the closure of dumping wastes into the Portman Bay (source: www.greenpeace.org).

2.3. Impacts of mining activities in the environment

Parallel to the development of mining activities strong environmental impacts took place. García-García (2004) estimated on $\sim 10 \text{ km}^2$ the total area directly impacted by mining activities in the local mining district. This stands for approximately 20 % of the total surface of the Mining District, but it does not include those surrounding areas affected by mining wastes such as salt marshes of the Mar Menor lagoon or agricultural fields (Figure 2.8).

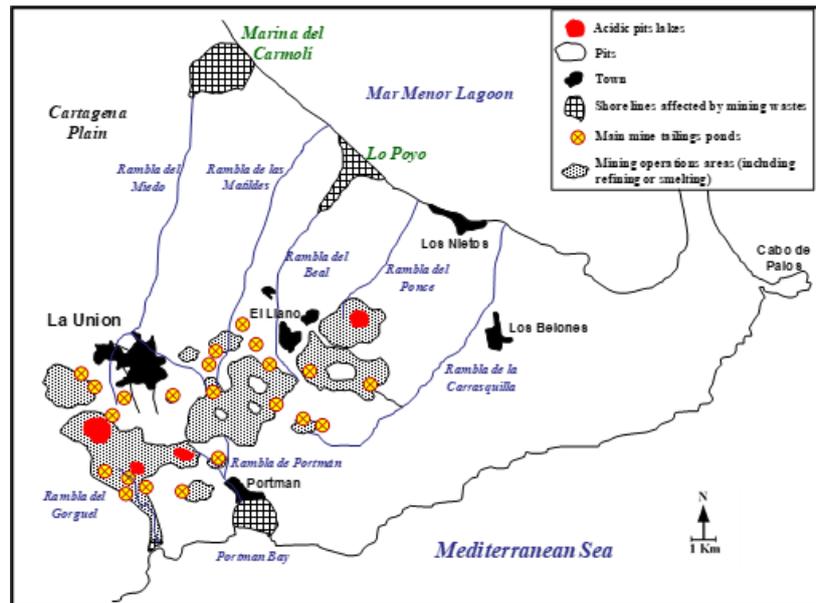


Figure 2.8: Mining impacts in the Cartagena-La Unión area (Párraga-Aguado (2015) adapted from Conesa and Schulin (2010)).

The modifications in the landscape caused by mining activities transformed the natural area into an industrial portrait, which included the modification of the orography, variation in the watercourses and the removing of the vegetation (Figure 2.9). As it occurred in other mining areas in Spain such as Almadén (Ciudad Real) or Rio Tinto (Huelva) the nuances of the mining landscape should be interpreted not only in terms of aesthetic values, but also including a social-historical view, since it represents the work of many generations and the legacy of the local communities (Conesa, 2010).



Figure 2.9: Picturesque portrait of the Mining District close to the El Gorguel Rambla.

Due to the high heterogeneity of mining wastes, García-García (2004) performed a classification based on their typology/source (Table 2.1).

Table 2.1: Summary of mining wastes. Source: García-García (2004).

Type of wastes	Number of structures	Area (km ²)	Volume (Mm ³)
Mine spoils (<i>escombreras</i>)	32	4.21	136
Mine tailings	89	2.18	23
Mine wastes in sea	3	0.83	25
Gravimetric spoils	119	0.65	3.73
Underground spoils	176	0.48	3.01
Gossans	11	0.26	6.93
High size wastes	1	0.06	0.59
Smelting wastes	19	0.13	0.66
Excavations materials from wells	1902	0.02	0.51
Total:	2352	8.82	199

In this classification, the highest surface is occupied by mine spoils or *escombreras* (Figure 2.10). These are structures of non-consolidated materials coming from open cast mines. They are formed by rocks with low metal ore concentrations and no interest in refining. The main problems of these structures come from their erosion due to their steep slopes but do not represent a risk in terms of containing high concentrations of metal(loid)s (Conesa and Schulin, 2010).



Figure 2.10: A former *escombrera* that has been spontaneously colonized by *Pinus halepensis*.

Several types of wastes, including gravimetric and underground spoils, gossans, high size wastes and smelting wastes cover an area smaller than 2 km². Their main environmental problems may come from their landscape impacts or their punctual risk for generating acid mine drainage. In the specific case of smelting wastes there is an on-going research because of being a potential source of radioactive emissions (González, 2019). Finally, mine tailings, although they show lower area than mine spoils, are of higher environmental concern as it was previously explained at the Section 1.1. Until 1955, the wastes of the metal ore refining activities were directly dumped into the ramblas. The accumulation of sediments in their riverbeds (Figure 2.11), led that year to prohibit direct dumping. Instead, mining refining facilities were obligated to construct tailings piles to dispose the wastes (Vilar and Egea-Bruno, 1990). There are about 89 of these structures spread across the Mining District (Figure 2.12). Most of them are perched on the slopes of the mountains and are coincidental with the headwater of the *ramblas* that drain the Sierra.



Figure 2.11: Riverbed filled with mining wastes of the El Beal Rambla at its headwater.



Figure 2.12: Belleza tailing located in the surroundings of La Unión town.

According to Conesa et al. (2008a), the local mine tailings can be categorized into two main classes: a) acidic pH tailings, which show pHs lower than 4 and exhibit high water soluble metal concentrations. Their mineralogical composition is characterized by the occurrence of reduced (e.g. sphalerite) and oxidised minerals (e.g. magnetite). These tailings are an important source of acid mine drainage; and b) neutral pH tailings, of pHs around 7, which show low soluble metal concentrations. Both types of tailings have common characteristics, which include a homometric texture (mainly composed by sandy or silt materials) and high salinity. Total metal(loid) concentrations are highly variable among tailings depending on the type of mineral ore. Several studies in the area characterized these concentrations which are provided at the Table 2.2.

Table 2.2: Metal concentrations reported by previous studies in the Cartagena-La Unión area and reference levels. Data are in mg kg⁻¹. "n.a." means not available.

Site	Reference	As	Cd	Cu	Pb	Zn	
Mine tailings	<i>Belleza</i> tailings pile	Conesa et al. (2008a)	1900	8.8	380	7000	5400
	<i>El Gorguel</i> tailings pile		350	34	84	5200	9100
	<i>El Lirio</i> tailings pile	Conesa et al. (2007)	n.a.	n.a.	150	4000	12000
Sediments of <i>ramblas</i> riverbeds	<i>El Gorguel</i>	Pavetti et al. (2006)	n.a.	n.a.	220-1700	6900- 18100	9000- 14400
	<i>La Carrasquilla</i>	García-García (2004)	n.a.	24	39	3700	3500
	<i>Ponce</i>		n.a.	25	30	1960	1990
	<i>Miedo</i>		n.a.	16	45	2250	2850
	<i>El Beal</i>	Robles-Arenas et al. (2006)	n.a.	15	35	950	650
	<i>El Beal</i>		n.a.	n.a.	n.a.	100000	10000
Salt marshes of the Mar Menor	<i>Lo Poyo</i>	Álvarez-Rogel et al. (2004)	n.a.	n.a.	110	8000	6900
	<i>Lo Poyo</i>	María-Cervantes et al. (2009)	500	9.1	75	7000	7100
	<i>Lo Poyo</i>	Conesa et al. (2011)					
	<i>Marina del Carmolí</i>	Jiménez-Cárceles et al. (2008)	700	80	400	16800	62000
Sediments in the Mar Menor		Sanchiz et al. (2000)	n.a.	0.3-0.6	n.a.	30-90	130
		Marín-Guirao et al. (2005)	n.a.	7	n.a.	10000	7000
		Robles-Arenas et al. (2006)	n.a.	10.000	200	10000	10000
Mediterranean coast	<i>Portmán</i> Bay	García et al. (2003)	n.a.	n.a.	150	8000	20000
	<i>El Gorguel</i> beach	Pavetti et al. (2006)	n.a.	n.a.	1050	2260	20400
Agricultural areas	Crop land near mine tailings	Conesa et al. (2010)	n.a.	n.a.	21	200-500	200-900
	Crop land close to La Unión	Conesa (2003)			77-160	28-150	93-400
Local forest areas	Regional Park of <i>Calblanque, Monte de las Cenizas y Peña del Águila</i>	Párraga-Aguado et al. (2013)	430	15	100	5400	7800
Reference levels	Geochemical backgrounds in the nearby Cartagena plain	Martínez-Sánchez and Pérez- Sirvent (2007)	7.0	0.32	12,6	9,3	41,4
	Environmental thresholds in the nearby Cartagena plain		16	0.5	30	57	90

To date, most of tailings in the area remain without being restored. Since tailings surfaces remain bare, erosion is able to spread their metal(loid) enriched particles to the surroundings. This has increased the concerns on the environmental health risks associated to tailings, which currently become an emerging issue in the local social context. These results are critical when tailings are located close to populations, where there is a toxicological risk for inhabitants

(Sánchez Bisquert et al., 2017). For those tailings far from populations, although they might not affect people directly, there is still a risk of transferring pollutants to the environment. Several studies (referenced in Table 2.2) have detected the presence of high metal(loid) concentrations coming from tailings in salt marshes of the Mar Menor lagoon (e.g. María-Cervantes et al., 2009) or in its sediments (e.g. Marín-Guirao et al., 2005), crop fields (Conesa et al., 2010) or local forests (Párraga-Aguado et al., 2013) (Figure 2.13). The main issues in these places come from the risk of metal transfer into food chain.



Figure 2.13: Presence of mine tailings close to local forests (left) and crop areas (right).

2.4. The specific area studied in this PhD Thesis

This PhD Thesis is focused on a specific mine tailings disposal site (37° 36' 15'' N, 0° 50' 06'' W) and their surroundings included in the Natura 2000 Site ES6200001 and Regional Park of *Calblanque, Monte de las Cenizas y Peña del Águila*. This is considered as Mining Heritage, being protected as *BIC* in the *Sierra Minera de Cartagena and La Unión (Sector VII)*. There are five main mine tailings piles in this site (Figure 2.14). One of them is covered by natural vegetation. In another one (*Agustín*), a pilot environmental restoration intervention has been led by the Sierra Minera Foundation and ANSE association as a part of the implementation actions of the Life + "Habitat Conservation 9570*, *Tetraclinis Articulata* Forests in the European Continent" Project 2018.

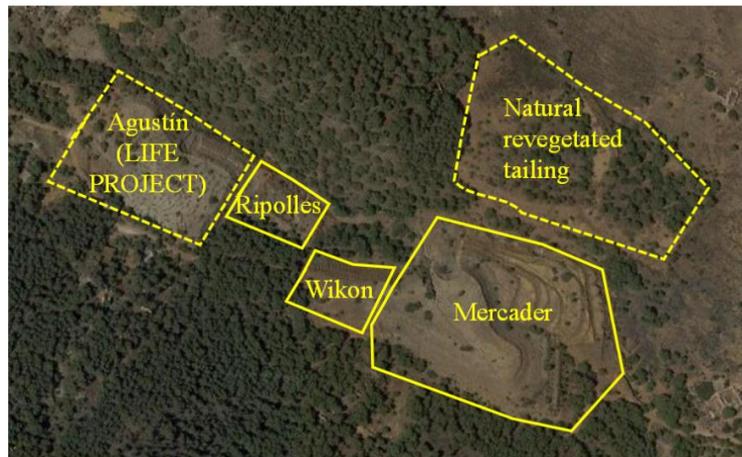


Figure 2.14: Situation of the studied tailings selected for this PhD Thesis. Pictured modified from Google Earth.

The other three tailings piles (*Ripolles*, *Wikon* and *Mercader*; Figure 2.14) are included in the National Inventory of Sludge Deposits from Treatment Processes of Extractive Industries (IGME, 2002). Based on this report, these tailings piles accumulate 940,000 m³ wastes and are considered as strongly affected by geo-structural instability and surface erosion (presence of gullies and collapsed walls). Thus, they act as a source of metal(loid) enriched particles for their surroundings (Párraga-Aguado et al., 2013; Navarro-Cano et al., 2018), which include the headwater of the *Carrasquilla Rambla* and a local forest area. The dry bed of the *rambla* is silted up by sediments from the tailings piles. These sediments, containing high metal(loid) concentrations, are carried out during intensive rainfalls to the Mar Menor lagoon (Conesa and Jiménez-Cárceles, 2007). In addition, the tailings piles are surrounded by a Mediterranean forest which is also included within the main distribution area of the European priority habitat 9570*. This habitat shapes an Iberian-North (*Pinus halepensis* - *Tetraclinis articulata*) mixed forest with abundant local endemic vegetation (Oreja et al., 2020). It is strongly impacted by high metal(loid) concentrations (Párraga-Aguado et al., 2013, 2014), which has resulted in a high ecotoxicological risks for biota (González-Alcaraz and van Gestel, 2015). After mining activity was discontinued 30-40 years ago, the spontaneous plant colonization of some patches of the tailings has been taking place (Párraga-Aguado et al., 2013, 2014). This provides an extraordinary natural laboratory for understanding the successional edaphic processes which occur in these systems.

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CHAPTER 3. Background and objectives

3.1. Background and objectives

Previous studies on environmental issues in the mining area of Cartagena-La Union (Table 3.1) have revealed the critical impacts due to mining activities (reviewed by Conesa and Schulin, 2010). Those research studies can be divided into two main categories: a) studies on characterization of contamination, including geochemistry, and assessment of metal(loid)s accumulation into biota; and b) research on environmental restoration.

This PhD thesis is oriented to environmental restoration, specifically focused on the implementation of phytomangement as a technique to decrease environmental risks associated to tailings.

Table 3.1: Scientific environmental studies related to mine tailings at the Cartagena-La Unión Mining District.

Topic	References
Field surveys on soil and plant metal(loid) content	Conesa et al. (2006; 2007a)
	Pérez-Sirvent et al. (2012)
	Martínez-López et al. (2014)
	Párraga-Aguado et al. (2014b)
Field studies on plant communities	Conesa et al. (2007b)
Field surveys on edaphic gradients and successional processes	Navarro-Cano et al. (2018; 2019)
	Oreja et al. (2020)
	Párraga-Aguado et al. (2013; 2014b; 2014c)
Effects of amendments on mine tailings properties	
Field experiments	Zanuzzi et al. (2009)
	Zornoza et al. (2012)
	Zornoza et al. (2013)
Pot experiments	Clemente and Bernal (2006)
	Pardo et al. (2011)
Effects of amendments on tailings and plant metal uptake	
Field experiments	Clemente et al. (2012)
	Kabas et al. (2012)
	Pardo et al. (2014b)
	Zornoza et al. (2015)
Pot experiments	Conesa et al. (2007c; 2007d; 2009)
	González-Alcaraz et al. (2011)
	Martínez-Oró et al. (2017; 2019)
	Pardo et al. (2014a)
	Párraga-Aguado et al. (2015)
Role of soil microorganisms	Carrasco et al. (2009; 2010)
	Zornoza et al. (2015)
	Colin et al. (2019)

Phytomanagement of mine tailings must deal with unfavourable edaphic conditions, which constraint plant growth (e.g. Conesa et al., 2006, 2007a). Among them, most authors highlighted metal(loid) concentrations and extreme pHs as the main issues for plant species establishment (Wong, 2003). However, plants growing at tailings in semiarid areas must deal with other additional constraints such as salinity, drought or low organic matter concentrations (low fertility) (Conesa et al., 2007a; Carrasco et al., 2010). Although the critical role of these parameters for plant growth had been suggested (Mendez and Maier, 2008), some studies obviated their combined effects on plant performance (Conesa et al., 2011, 2014). In addition, the heterogeneous nature of tailings conditioned the occurrence of edaphic gradients at the tailings surfaces, which in turn, generated edaphic niches with specific conditions for plant growth (whether more or less favorable) (Párraga-Aguado et al., 2013). Párraga-Aguado et al. (2013) showed that some factors, such as electrical conductivity or texture, played a more important role in determining the distribution of pioneer vegetation than metal(loid) concentrations at the neutral pH tailings of the Cartagena-La Unión mining area. The pioneer vegetation was mostly composed by ruderal plant species, some of them with halophytic characteristics, which were able to colonize high saline patches of the tailings (Párraga-Aguado et al., 2013, 2014a). The establishment of pioneer plant species in bare areas triggered edaphic successional processes at their rhizospheres due to the improvement of soil fertility and microbial biomass (Párraga-Aguado et al., 2014b), making possible the establishment of late successional species that were not able to grow in the initial bare surfaces of the tailings. The associations between pioneer and late successional plant species formed vegetated patches at the tailings also known as fertility islands. The occurrence of fertility islands is considered a key factor to achieve a self-sustaining vegetation (Párraga-Aguado et al., 2014c). One of the critical keys in these systems is to understand the role of microbial communities in the aforementioned successional edaphic processes at metals polluted sites (Moynahan et al., 2002; Li et al., 2015; Colin et al., 2019). For instance, the early stages of edaphic succession at recently built mine tailings might be driven by primer lithotrophic microbial colonizers that play an important role in altering soil mineralogy and geochemistry (Colin et al., 2019). The establishment of spontaneous vegetation may

reconfigure the structure of microbial communities to more organotrophic oriented groups, in which new nutrient resources coming from plants may open new ecological niches and set the basis for a self-sustaining system, in terms of biogeochemical cycles (Huang et al., 2011). Some studies have inquired about the effects of specific soil properties on the soil microbial communities structure, including factors such as pH (Rousk et al., 2010), organic matter (Quadros et al., 2016) or the role of plant rhizospheres as key factors for microbial diversity and composition (Colin et al., 2019). However, it is not well understood the combined effect of several limiting edaphic factors (e.g. metals, organic matter, salinity, plant rhizospheres) on microbial composition during the successional edaphic processes, as it occurs in metal(loid) impacted ecosystems (Wakelin et al., 2012; Azarbad et al., 2015;). To date, studies on microbiology at the Sierra Minera of Cartagena-La Unión have been focused on the microorganisms productivity after amendments application (Zornoza et al., 2015), descriptions of microbial communities structure by PLFA analysis, determination of soil enzymatic activities (Carrasco et al., 2010; Zornoza et al., 2015) or the inoculation of specific microbial taxa to promote the establishment of specific plant species in mesocosm experiments (Carrasco et al., 2009). Recently, Colin et al. (2019) performed a research based on molecular biology tools, and was able to determine the bacterial composition associated to different plant species growing at local tailings. However, there was not a comprehensive study on how edaphic factors conditioned the microbial composition at tailings and its changes during the edaphic successional processes triggered by pioneer vegetation.

Previous plant surveys at the mining area of Cartagena-La Unión have selected suitable candidates for phytostabilization (Conesa et al., 2006, 2007a; Pérez-Sirvent et al., 2012; Martínez-López et al., 2014). Most of these studies rely on the ability of plants to cope with the limiting edaphic factors of the tailings and on their metal uptake capabilities. The goal was to select those plant species with lower metal(loid) transfer into shoots in order to avoid risks of pollutant accumulation into the food chain. In most cases, low metal(loid) concentrations in the above-ground parts were reported for the plant species studied at the local tailings (e.g. $<10 \text{ mg kg}^{-1}$ Cu, $<200 \text{ mg kg}^{-1}$ Pb, $<500 \text{ mg kg}^{-1}$ Zn; Conesa and Schulin, 2010). Párraga-Aguado et al. (2014b)

and Martínez-Oró et al. (2017; 2019) performed an ecological approach for tailing piles phytostabilization, focusing on the behavior of plant species combinations to facilitate/stimulate plant establishment at tailings. Recent reports on mine tailings phytomanagement have pointed out the importance of soil microbiology for the establishment of self-sustainable vegetation (Kolaříková et al., 2017; Sun et al., 2018). In turn, soil microbial composition is highly dependent on the specific rhizosphere microbiome. This plant species-microbial community dependence is influenced by root exudates or plant litter, which differ in their composition among plant species (Deng et al., 2018; Sun et al., 2018). Therefore, an important query in these systems is to determine the relationship between edaphic parameters and the specific rhizosphere microbiome during plant establishment at mine tailings. To date, there are not studies at the mining area of Cartagena-La Union that include a specific characterization of plant species microbiome and the consequences that this specificity microbiome-plant species may have in the final plant establishment.

The use of amendments is a suitable tool to improve the deficient edaphic conditions of mine tailings and accelerate the establishment of vegetation (Clemente et al., 2010; Kohler et al., 2014; Párraga-Aguado et al., 2015). This topic has been widely studied in phytomanagement projects, including at the Cartagena-La Unión area (e.g. Clemente and Bernal, 2006; Conesa et al., 2007d; González-Alcaraz et al., 2011; Pardo et al., 2011, 2014a, 2014b; Párraga-Aguado et al., 2015; Zornoza et al., 2015, Table 3.1). These studies covered a broad variety of amendments whether organic and/or lime materials, which were tested either in pots, mesocosms or field plots. The effects on soil fertility, plant growth, metal(loid) availability and/or plant metal uptake were evaluated. Recently, Párraga-Aguado et al. (2015) and Martínez-Oró et al. (2019) carried out mesocosm experiments to evaluate how organic amendments altered/modulated the ecological interactions at both, intraspecific and interspecific levels, in local plant species. However, there is a lack of understanding of the amendment effects on microbial composition at middle term. It is important to assess this because of the autochthonous microbiology, present at tailings and already adapted to metals and low fertility conditions, may be negatively impacted by those

microorganisms contained in the amendments or by the changes in soil conditions that amendments may generate (Grandlic et al., 2008).

The general purpose of this PhD Thesis was to increase the knowledge on the relationship between edaphic and microbiological factors involved in the spontaneous plant colonisation of mine tailings. This will improve the feasibility of phytomanagement in terms of phytostabilization applied to these environments in a semiarid climate context. The specific objectives were:

Objective 1- To elucidate which factors, including edaphic, vegetation and microbiological aspects, determine soil functionality in the phytomanagement of mine tailing piles in semiarid areas.

Objective 2- To identify the edaphic factors, including microbiology, affecting the establishment of plant species with contrasting growth patterns during the phytomanagement of a mine tailings pile.

Objective 3- To assess the effects of soil organic amendments on the microbial composition (bacteria and fungi) of mine tailings.

Objective 4- To assess the effects on microbial composition (bacteria and fungi) of the presence of plants and litter in amended/non-amended tailings.

In order to achieve these goals, this PhD thesis has been divided in two parts (Figure 3.1) corresponding to 1) field data (Chapters 4 and 5) and 2) mesocosm experiments carried out in climate chamber at the IBV-UPCT (Chapters 6 and 7).

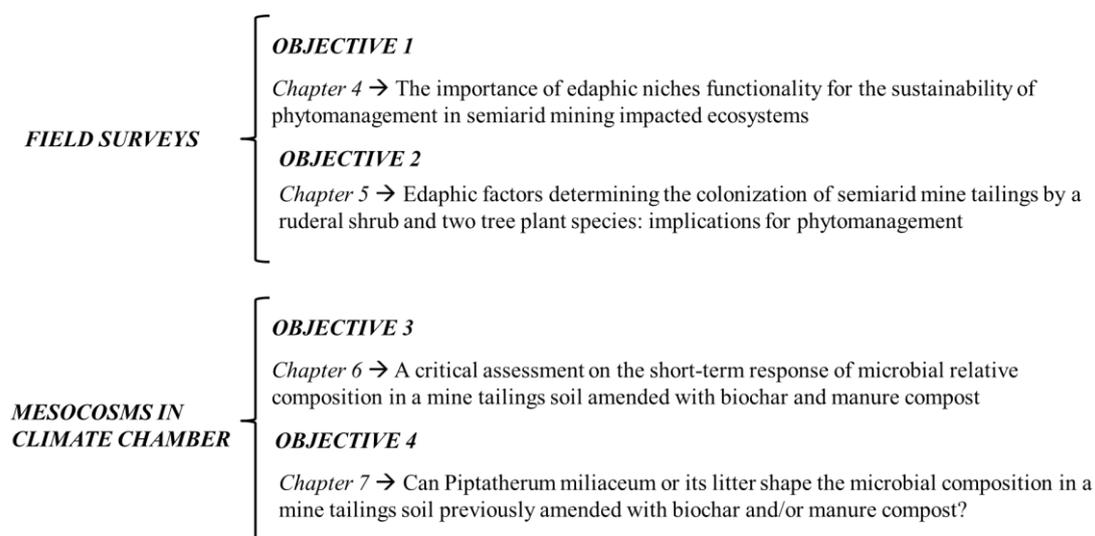


Figure 3.1: Connection between the objectives and the Thesis' chapters.

Chapter 4, focused on the Objective 1, includes a field study using a transect experimental design in a former mining area located in southern Spain. Soil characteristics, including edaphic parameters, plant biodiversity and bacterial and fungal compositions, were determined.

Chapter 5, focused on the Objective 2, includes a field survey study. Soil parameters and microbial composition in the rhizospheres of several plant species from tailings were studied.

Chapter 6, focused on the Objective 3, shows a mesocosm experiment in which soil parameters and microbial composition were analysed after adding a biochar and/or manure amendment.

Chapter 7, focused on the Objective 4, includes a mesocosm experiment where the effects on the soil microbial composition associated to *Piptatherum miliaceum* and its litter were studied in amended and non-amended mesocosms.

3.2. References

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**CHAPTER 4. The importance of edaphic niches functionality
for the sustainability of phytomanagement in semiarid mining
impacted ecosystems**

**The importance of edaphic niches functionality for the sustainability of
phytomanagement in semiarid mining impacted ecosystems**

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Abstract

Mining activities on metal sulphide ores are considered one of the most environmentally damaging anthropogenic activities worldwide, mainly due to the production of metal(loid)-enriched tailings. The objective of the work was to elucidate which factors, including edaphic, vegetation and microbiological aspects, determine soil functionality in the phytomanagement of mine tailing piles in semiarid areas. For this purpose, a field study was conducted using a transect experimental design in a former mining area located in southern Spain. Soil characteristics, including edaphic parameters, bacterial and fungal compositions, were determined. The variation in edaphic parameters (pH, electrical conductivity, organic carbon, particle size distribution, etc) through the deterioration transect caused a decrease in plant and microbial diversities, as well as in microbial productivity measured by enzymatic activities. Variation in bacterial relative abundances through the transect was better explained by soil fertility related factors, such as organic matter, pH, salinity, enzymatic activities or microbial biomass (which in turn were related to the presence of plants) than by metals concentrations. The presence of vegetation at the tailings allowed the transition from lithotrophic bacterial orders, which dominated in bare tailings areas (e.g. *Acidimicrobiales*, *Chromatiales*) to organotrophic oriented orders (e.g. *Cytophagales*, *Actinomycetales*). Vegetated patches at the tailings pile and its borders shared some organotrophic bacteria with control forest samples (e.g. *Rhizobiales*), indicating that natural plant mediated successional processes might stimulate biogeochemical cycles similar to those occurring at non-polluted systems. This functionality of the impacted sites, which include unfavourable edaphic conditions, should be taken into account in the phytomanagement of mine tailings, since it may support its long-term sustainability. Unlike bacteria, variations in fungal relative abundances through the transect were not clearly explained by soil parameters or the presence of plants. Other factors related to spatial distribution or the type of organic substrates may be more determinant in the case of fungi.

4.1. Introduction

Mining activities on metal sulphide ores are widely considered one of the most environmentally damaging activities worldwide (e.g. Wong, 2003; Li et al., 2015). Among impacts of this type of mining, mine tailings piles (left over from mining refining operations) concentrate the highest risks for environmental health. They consist of structures of non-consolidated materials with extreme pH values, high salinity and high metal(loid) concentrations, which normally leave their surfaces bare and prone to erosion (Mendez et al., 2008; Li et al., 2015). Attempts to remediate these sites through removal operations require high economic investment, and for this reason *in-situ* alternatives are normally proposed by environmental authorities (Conesa and Schulin, 2010). Among them, phytomanagement by phytostabilization has been raised as an ecological and more economic option, consisting of improving a self-sustaining vegetation cover for stabilizing the tailings pile's surface and preventing them from erosion (Rosario et al., 2007; Robinson et al., 2009). However, phytomanagement of tailings must deal with some constraints. Harsh conditions of tailings may prevent vegetation from colonization and thus plant selection must be restricted to tolerant plant species. Previous research on revegetation of tailings has revealed that soil properties related to fertility (e.g. pH, electrical conductivity or organic matter) may play a more important role in plant establishment than metal(loid)s tolerance issues. Therefore, the understanding of dynamics in the evolution of edaphic factors and successional processes in the soil are critical to achieve a self-sustaining vegetation (Párraga-Aguado et al., 2013).

Metal impacted ecosystems, such as those found in mining areas, develop specific microbial communities, which not only are able to exhibit tolerance or resistance to contaminants but also may sustain biogeochemical processes under harsh environmental conditions not tolerated by most higher organisms (Huang et al., 2011; Fernandes et al., 2018). This makes soil microbiology a critical factor to be taken into account in the restoration of mining impacted soils for two main reasons: 1) microbes ability to interact with plants rhizospheres, acting as filters to

deplete metals phytotoxicity and, 2) microbes as supporters of C-N cycles providing available nutrients for plant growth (Thavamani et al., 2017).

There are many studies dealing with the characterization of microbial communities (bacteria and fungi) in metal impacted ecosystems (e.g. Hu et al., 2007; Fernandes et al., 2018). Some studies have focused on the comparison of microbial communities between polluted and non-polluted areas (Rosenfeld et al., 2018) or among sites with different stages of environmental restoration within the same region (Quadros et al., 2016), in order to determine site specific resistant microbial communities. However, one of the critical keys in these systems is understanding the role of microbial communities in successional edaphic processes, which occur in metals polluted sites (Moynahan et al., 2002; Li et al., 2015; Colin et al., 2019). For instance, the early stages of edaphic succession at recently built mine tailings might be driven by primer lithotrophic microbial colonizers that play an important role in altering soil mineralogy and geochemistry (Colin et al., 2019). The establishment of spontaneous vegetation may reconfigure the structure of microbiological communities to more organotrophic oriented groups, in which new nutrient resources coming from plants may open new ecological niches and set the base for a self-sustaining system in terms of biogeochemical cycles (Huang et al., 2011).

On the other hand, it is important to assess common operations in the restoration of tailings, such as the addition of soil conditioners or the introduction of exogenous plant species, because the autochthonous microbiology, adapted to metals and low fertility conditions, may be negatively impacted (Grandlic et al., 2008). Moreover, mine tailings provide an advantageous environment to study microbial interactions with gradients of geochemical soil properties (e.g. Pennanen, 2001; Pereira et al., 2014). Advanced studies have looked at the effects of specific soil properties on the structure of soil microbial communities, including factors such as pH (Rousk et al., 2010), organic matter (Quadros et al., 2016) or the role of plant rhizospheres as drivers for microbial diversity and composition (Colin et al., 2019). However, it is not well understood the effect of combined factors (e.g. metals, organic matter, salinity, plant rhizospheres) on microbial

composition during the successional edaphic processes, which occur in metal(loid) impacted ecosystems (Wakelin et al., 2012; Azarbad et al., 2015).

The objective of the work was to elucidate which factors, including edaphic, vegetation and microbiological aspects, determine soil functionality in the phytomangement of mine tailing piles in semiarid areas. As a specific goal, it was assessed how the edaphic gradients affect the structure of the microbiological communities (bacteria and fungi). We hypothesised that microbial diversity may be more negatively impacted by metal(loid) concentrations than by other secondary soil stressors, such as salinity or low fertility. For this purpose, a field study was conducted by using a transect experimental design in a former mining area located in southern Spain. Soil characteristics, including edaphic parameters, plant biodiversity and bacterial and fungal compositions, were determined.

4.2. Material and Methods

4.2.1. Study site

The study site, *Peña del Águila*, (0-385 m a.s.l.; 37° 37' N, 0° 49' W– 37° 35' N, 0° 50' W, ~50 km²) is located in the southeast of Spain and belongs to the former Mining District of Cartagena-La Unión (Figure 4.1). Former mining operations extracted metallic sulphur minerals such as galena, pyrite or sphalerite. The climate of the zone is semiarid with annual rainfall of 250-300 mm, mainly during spring and autumn, and an annual average temperature of 18°C. Further information on environmental impacts of the mining activity is available in Conesa and Schulin (2010).

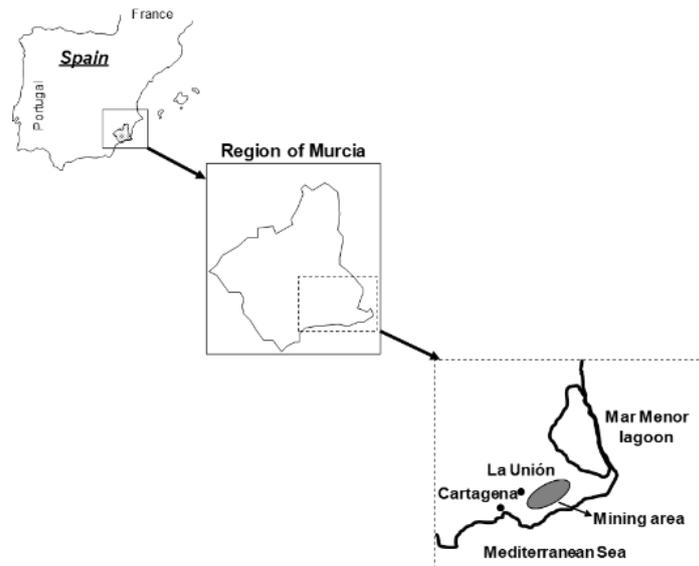


Figure 4.1: Location of the studied area.

The sampling area is located at a former mine tailings pile, discontinued forty years ago, which is surrounded by a Mediterranean Aleppo pine forest landscape composed of tree species including *Pinus halepensis*, *Tetraclinis articulata* or *Olea europaea* and xerophytic shrubs, such as *Pistacia lentiscus*, *Rosmarinus officinalis* or *Calicotome intermedia*.

4.2.2. Sampling collection

A transect of deteriorated soil (Figure 4.2 for scheme and Table 4.1 for plant species inventory) was selected for soil sampling during spring of 2018 from a control reference area to the mine tailings pile's plateau. This transect provided the following microenvironments: A) a forested site (*Control*), which consisted of a non-mine impacted area covered by a typical Mediterranean forest located at 2 km from the mine tailings pile; B) a transitional area consisting of a mine impacted forest with similar plant inventory to the *Control* but located at the external border of the mine tailings pile (*External Border*); C) a site which included the tailings substrate at the lowest part of the mine tailings pile's slope (*Internal Border*); D) a site within the tailings zone, which consisted of rhizospheric soil samples from spontaneous coniferous trees (*P. halepensis*) growing at the tailings pile's slope (*Slope Trees Rhizospheres*); E) bulk tailings substrate samples from the plateau (*Bulk Tailing*); and F) which consisted of soil samples from vegetated patches at the mine tailings pile's plateau (*Fertility Islands*).

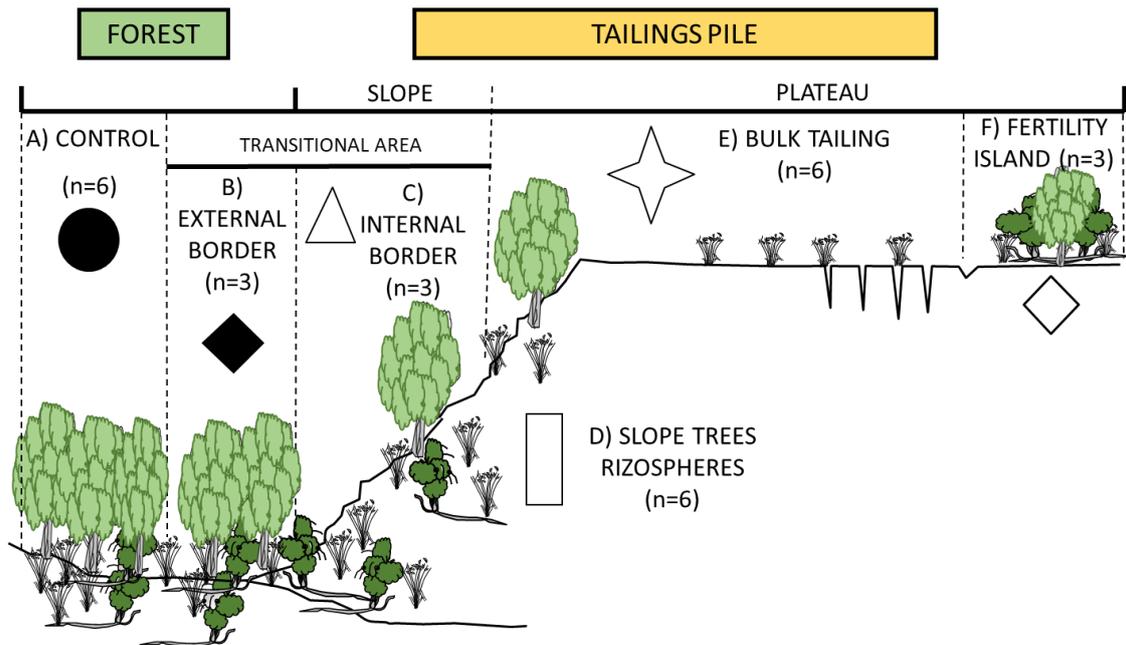


Figure 4.2: Scheme of the sampling transect. Symbols for each sampling site are employed in the PCA and CCA figures through the manuscript. “n” represents number of soil samples replicates at each site of the transect.

At each microenvironment, three experimental plots of 5 x 5 m² were performed, where plant ecological parameters were recorded (species and number of individuals per specie) and soil samples taken. One or two composite samples, composed of three sub-samples, were taken from each plot resulting in three replicates for zones B, C and F and six replicates for A, D and E (Figure 4.2). The different number of replicates for separate zones was based on the relative area covered by each zone. An aliquot of each composite sample was stored in sterile falcon tubes, kept in ice for transport to the lab, sieved through a 2 mm screen and stored at -20°C until enzymatic and microbial analysis. Soil samples for edaphic characterization were air dried at room temperature, sieved through a 2 mm screen, homogenised and stored in sealed plastic bags prior to lab analytical procedures.

Table 4.1: Plant species inventory following the experimental transect.

Plant species	Family	Forest		Mine Tailings			
		Control	External Border	Slope		Plateau	
				Internal Border	Slope Trees	Bulk Tailing	Fertily Islands
<i>Atriplex halimus</i> L.	Chenopodiaceae					X	
<i>Arisarum vulgare</i> Targ.-Tozz.	Araceae		X				
<i>Asparagus</i> sp.	Liliaceae	X	X				X
<i>Atractylis humilis</i> L.	Asteraceae	X	X				
<i>Ballota hirsuta</i> Bentham	Labiatae						
<i>Brachypodium retisum</i> (Pers.) Beauv.	Gramineae	X	X				
<i>Bupleurum fruticosum</i> Loeff. ex L.	Umbelliferae		X				
<i>Calicotome intermedia</i> (C. Presl.)	Leguminosae	X					
<i>Chamaerops humilis</i> L.	Palmae	X	X	X			
<i>Cheirolophus inybaeus</i> (Lam.) Dostál	Asteraceae		X				
<i>Cistus monspeliensis</i> L.	Cistaceae			X			
<i>Convolvulus arvensis</i> L.	Convolvulaceae		X				
<i>Dittrichia viscosa</i> (L.) Greuter	Asteraceae		X	X	X		
<i>Dorycnium pentaphyllum</i> Scop.	Leguminosae		X		X		
<i>Eryngium campestre</i> L.	Umbelliferae	X	X				
<i>Fumana ericoides</i> (Cav.) Gand	Cistaceae	X					
<i>Lavandula dentata</i> L.	Labiatae		X				
<i>Helianthemum almeriense</i> Pau in Mem. Mus	Cistaceae	X					
<i>Helianthemum syriacum</i> (Jacq.)	Cistaceae			X			
<i>Helichrysum decumbens</i> (Lag.) Cambess.	Asteraceae		X	X	X		X
<i>Hippocrepis ciliata</i> Willd.	Leguminosae	X					
<i>Lapiedra martinézii</i> Lag.	Amaryllidaceae		X				
<i>Limonium</i> sp.	Plumbaginaceae						X
<i>Lygeum spartum</i> L.	Gramineae				X		
<i>Nothoscordum inodorum</i> (Aiton) Nichol.	Liliaceae		X				
<i>Olea europaea</i> L.	Oleaceae		X		X		X
<i>Phagnalon saxatile</i> (L.) Cass.	Asteraceae		X	X			
<i>Phillyrea angustifolia</i> L.	Oleaceae		X				
<i>Pinus halepensis</i> Mill.	Pinaceae	X	X	X	X	X	X
<i>Piptatherum miliaceum</i> (L.) Cosson	Gramineae			X	X		
<i>Pistacia lentiscus</i> L.	Anacardiaceae		X	X	X		X
<i>Polygala rupestris</i> Pourret	Polygalaceae			X	X		
<i>Psoralea bituminosa</i> L.	Leguminosae			X			
<i>Rhamnus alaternus</i> L.	Rhamnaceae		X				
<i>Rhamnus lycioides</i> L.	Rhamnaceae	X	X				
<i>Rosmarinus officinalis</i> L.	Labiatae	X					
<i>Ruta angustifolia</i> Pers.	Rutaceae		X				
<i>Salsola kali</i> L.	Chenopodiaceae					X	
<i>Salsola oppositifolia</i> Desf.	Chenopodiaceae						X
<i>Satureja obovata</i> Lag.	Labiatae	X	X				
<i>Sedum sediforme</i> (Jacq.) Pau	Crassulaceae	X					
<i>Sonchus tenerrimus</i> L.	Asteraceae		X	X			X
<i>Stipa tenacissima</i> L.	Gramineae	X					
<i>Tamarix doveana</i> Bunge	Tamaricaceae						X
<i>Tetraclinis articulata</i> (Vahl) Mast.	Cupressaceae	X		X	X		
<i>Teucrium carthagenense</i> Lange	Labiatae	X	X		X		
<i>Teucrium pseudo-chamaepitys</i> L.	Labiatae		X				
<i>Thymus hyemalis</i> Lange	Labiatae	X	X				
<i>Thymelaea hirsuta</i> (L.) Endl.	Thymelaeaceae	X					
<i>Urginea maritima</i> (L.) Baker	Liliaceae		X				
<i>Zygophyllum fabago</i> L.	Zygophyllaceae					X	X

With the plant ecological data (Table 4.1), the Shannon-Weaver index (H') was calculated for estimating the heterogeneity of the plant species at each plot (Shannon and Weaver, 1963) as:

$$H'p = - \sum_{i=1}^S p_i \ln p_i$$

Where p_i is the relative frequency of the species “i” at each plot and $\sum p_i = 1$ and S is the number of plant species at each plot.

4.2.3. Analysis of soil physical and chemical properties

The pH and Electrical Conductivity (EC) were measured in 1:5 soil:water ratio extracts. These extracts were filtered through nylon membrane 0.45 μm syringe filters (WICOM) and used for measuring water extractable ions (Cl^- and SO_4^{2-}) and Dissolved Organic Carbon (DOC) by employing an Ion Chromatographer (Metrohm) and a TOC-automatic analyser (TOC-VCSH Shimadzu), respectively. Metal(loid)s mobility was assessed by a 1:2.5 soil:0.01M CaCl_2 extraction (González et al., 2011). Resulting extracts were analysed using an ICP-MS (Agilent 7500A) for metal(loid)s (As, Cd, Cu, Mn, Pb, Zn). Total Nitrogen (TN) was determined following the Kjeldahl method (U.S.D.A, 1996) and particle size distribution according to the method of Bouyoucos` densimeter proposed by Gee and Bauder (1986). Organic Carbon (OC) concentrations were obtained after oxidising organic matter by potassium dichromate. Total element concentrations were determined in ground sub-samples by X-ray fluorescence (Bruker S4 Pioneer).

Soil enzymatic activities, dehydrogenase (García et al., 1993) and β -glucosidase (Reboreda and Caçador, 2008), were measured using unaltered portions of soil samples stored at -20°C . Microbial Biomass Carbon (MBC, from now on) was determined by the measurement of the extractable organic carbon by 0.5 M K_2SO_4 after a 24h CHCl_3 -fumigation (Vance et al., 1987; Wu et al., 1990) employing a TOC-automatic analyser (TOC-VCSH Shimadzu).

4.2.4. DNA extraction, PCR amplification and sequencing

Microbial (bacteria and fungi) DNA was extracted from 0.25 g soil using PowerSoil DNA Isolation Kit (MOBIO), according to the manufacturer's instructions. The isolated DNA was quantified using a NanoDrop 2000 spectrophotometer. Metagenomic analysis was performed at the Genomic Unit from the Centre for Genomic Regulation (Genomic Unit-CGR Core Facilities, Barcelona, Spain). Two separate PCR reactions per sample were performed in order to detect bacterial 16S rDNA amplification (V3-V4 region) and fungal internal transcribed spacer 2 (ITS2) amplification. Each PCR amplification were carried out on 4 μl DNA, 0.2 μM of each forward

and reverse primer and Kapa ready mix (Kapa biosystems) in a total volume of 10 μ l. The 16S rRNA V3V4 region was amplified as previously described (Willis et al., 2018) using a set of modified primers V3-V4-F and V3-V4-R that contains a 1-4 bp “heterogeneity spacer”, which was designed by the Centre for Genomic Regulation (Genomic Unit), to mitigate the issues caused by low sequence diversity amplicons. PCR conditions were modified: an initial denaturation at 95°C for 3 min, followed by 25 three-step cycles consisting of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s; and a final 5 min extension at 72°C. The ITS2 region from DNA sample extracts were amplified using forward primer ITS3-KYO-F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGATGAAGAACGCAGCGAA-3') and reverse primer ITS4-R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCTCCGCTTATTGATATGC-3'). Samples were incubated in a thermocycler using a program of denaturing at 95°C for 3 min, followed by 35 three-step cycles consisting of 98°C for 20 s, 50°C for 30 s and 72°C for 30 s; and a final 5 min extension at 72°C.

After first PCR step, water was added to a total volume of 50 μ l and reactions were purified using AMPure XP beads (Beckman Coulter) with a 0.9X (V3-V4 amplicon) or 1.2X (ITS amplicon) ratio according to manufacturer's instructions. PCR products were eluted from the magnetic beads with 32 μ l of Buffer EB (Qiagen) and 30 μ l of the eluate were transferred to a fresh 96-well plate. The above-described primers contain overhangs allowing the addition of full-length Nextera adapters with barcodes for multiplex sequencing in a second PCR step, resulting in sequencing ready libraries. To do so, 5 μ l of the first amplification were used as template for the second PCR with Nextera XT v2 adaptor primers in a final volume of 50 μ l using the same PCR mix and thermal profile as for the first PCR but only 8 cycles. After the second PCR, 25 μ l of the final product was used for purification and normalization with SequalPrep normalization kit (Invitrogen), according to manufacturer's protocol. Libraries were eluted in 20 μ l volume and pooled for sequencing. Final pools were quantified by qPCR using Kapa library quantification kit for Illumina Platforms (Kapa Biosystems) on an ABI 7900HT real-time cycler (Applied

Biosystems). Sequencing was performed in Illumina MiSeq with 2×300 bp reads using v3 chemistry with a loading concentration of 18 pM.

Raw DNA sequences, from both Bacteria and Fungi, were processed at the Bioinformatics Unit from Centre for Genomic Regulation (Genomic Unit-CGR Core Facilities, Barcelona, Spain) using the Mothur software (version 1.39.5) (Schloss et al., 2009), according to the standard operating protocol (Kozich et al., 2013). Overlapping pairs of sequence reads were merged into contigs. In addition, reads with ambiguous bases, duplicated contigs and homopolymers longer than 13 bp were removed. The VSEARCH algorithm (embedded in the Mothur framework) was used to remove chimeras and these were subsequently omitted. The resulting sequences were classified according to the taxonomy into the corresponding Operational Taxonomic Units (OTUs) at 97 % similarity, besides using the reference trainset 16_022016.pds from https://mothur.org/wiki/RDP_reference_files for Bacteria and ITS sequences provided by the UNITE ITS database (version 7.2) at <https://unite.ut.ee/repository.php> for Fungi. Undesired lineages such as Bacteria, Plantae, Animalia, Protista, “unknown” and other were removed. The final sequences were then grouped again into OTUs, using the cluster command in Mothur, which was based on sequence similarity distances at the 0.03 and the 0.05 cut off level for Bacterial and Fungi, respectively. Finally, the resulting OTUs mapping to the same genus were grouped together.

Relative abundances for each taxon were calculated as a percentage to the total count of reads in each sample, and the average was calculated for each study area. Orders (for both bacteria and fungi) and genera (for bacteria only), which showed abundance higher than 1 % in at least two sampling sites were considered separately. For bacteria, phyla with relative abundances below 1 % in at least two microenvironments were grouped together in the category *Others*. For fungi, phyla with relative abundance below 1 % in six microenvironments studied were grouped in *Others*. For both bacteria and fungi, the Shannon-Index at genus level was calculated as it was described for plants above.

4.2.5. Data analysis

Statistical analyses were performed with the software IBM SPSS Statistics 24. Homogeneity of variances was tested using Levene's test and data were transformed as needed to fit to a normal distribution. Means for soil, plant and microbiological data were compared by one-way ANOVA with Tukey's test. Pearson's correlations between selected parameters were also performed. Soil data were analysed using Principal Component Analysis (PCA) for evaluating the changes of soil properties along the deterioration transect. Data on bacterial and fungal orders abundances were analysed by Canonical Correspondence Analysis (CCA) for evaluating the relationship between soil fertility and soil microorganisms. The CANOCO software for Windows v4.02 was used for both, PCA and CCA (Ter Braak and Smilauer, 1999).

4.3. Results

4.3.1. Edaphic parameters and enzymatic activities

The results of edaphic parameters and enzymatic activities (Table 4.2) grouped the samples into four groups which, following the deterioration transect, (from *Control* to the tailings pile) resulted in: 1) *Control*, 2) *External Border*, 3) *Internal Border* and *Slope Trees Rhizospheres*, and 4) *Bulk Tailing* and *Fertility Islands*. The first group, composed of the *Control* samples, were characterised by better soil fertility parameters (e.g. highest TN, lowest EC) and lower metal(loid) concentrations than the tailings' pile samples ($p < 0.05$ for clay and sand percentages, EC, enzymatic activities, MBC, OC, DOC, TN, total metal concentrations and 0.01 CaCl₂ extractable Mn and Zn concentrations). The second group included the *External Border* samples, which showed similar soil organic matter values to the *Control* samples (e.g. $p > 0.05$ for OC, DOC) but lower (e.g. $p < 0.05$ for OC, DOC and TN) than those obtained at the tailings pile areas. Compared to the *Control* samples, the *External Border* showed significant higher metal(loid) concentrations (e.g. $p < 0.05$, total concentrations and 0.01 CaCl₂ extractable Zn concentrations) and lower MBC and enzymatic activities values ($p < 0.05$). The third group, composed of those samples coming from the slope of the tailings pile (*Internal Border* and the *Slope Trees Rhizospheres*) showed,

compared to the *External Border*, similar metal(loid) concentrations ($p > 0.05$ for all total concentrations) but lower soil fertility indicators (e.g. $p < 0.05$, lower OC, DOC and enzymatic activities, higher EC and SO_4^{2-}). Finally, the fourth group included the tailings pile samples from the plateau, *Fertility Islands* and *Bulk Tailing*. The latter showed the most adverse soil fertility conditions ($p < 0.05$, highest EC, lowest MBC and enzymatic activities). Compared to the *Bulk Tailing*, the *Fertility Islands* showed lower EC ($p < 0.05$), higher organic content and enzymatic activities values (e.g. $p < 0.05$ for OC, DOC, MBC and enzymatic activities) but similar total and 0.01 CaCl_2 extractable metal concentrations ($p > 0.05$ for all metals).

Table 4.2: Results of soil analyses for the transect samples. “1:5” refers to the results of the 1:5 soil water extract. Data are average \pm standard error. Different letters among columns of the same row indicate significant differences (ANOVA with Tukey’s test, $p < 0.05$). Number of replicates are 3 or 6.

Parameter	Units	Forest				Tailings				Plateau				
		Control	External Border	Internal Border	Slope	Trees	Rhizospheres	Bulk Tailings	Fertility Islands					
Edaphic parameters														
pH (1:5)		7.97 \pm 0.03 b	7.92 \pm 0.03 b	7.39 \pm 0.01 a	7.36 \pm 0.06 a	7.36 \pm 0.06 a	7.15 \pm 0.03 a	7.53 \pm 0.21 a						
Electrical Conductivity (EC) (1:5)	dS m ⁻¹	0.25 \pm 0.02 a	0.26 \pm 0.02 a	2.69 \pm 0.09 bc	1.66 \pm 0.37 b	1.66 \pm 0.37 b	3.31 \pm 0.16 c	1.44 \pm 0.62 b						
Organic Carbon (OC)	g kg ⁻¹	24.0 \pm 2.3 c	21.7 \pm 1.1 c	3.1 \pm 0.4 a	5.4 \pm 0.7 ab	5.4 \pm 0.7 ab	4.3 \pm 0.4 a	8.0 \pm 0.7 b						
Dissolved Organic Carbon (DOC) (1:5)	mg kg ⁻¹	202 \pm 45 d	181 \pm 22 d	23 \pm 1 ab	44 \pm 5 bc	44 \pm 5 bc	16 \pm 2 a	70 \pm 9 c						
Total Nitrogen (TN)	g kg ⁻¹	2.42 \pm 0.18 c	1.47 \pm 0.09 b	0.12 \pm 0.02 a	0.27 \pm 0.05 a	0.27 \pm 0.05 a	0.08 \pm 0.01 a	0.46 \pm 0.13 a						
Particle size distribution	%	27.7 \pm 0.9 c	2.8 \pm 0.8 b	1.0 \pm <1 a	1.7 \pm 0.7 ab	1.7 \pm 0.7 ab	1.0 \pm <1 a	1.6 \pm 0.5 ab						
clay		32.3 \pm 1.2 c	30.9 \pm 2.3 c	3.5 \pm 1.3 a	13.0 \pm 1.8 b	13.0 \pm 1.8 b	19.3 \pm 4.2 b	16.6 \pm 1.1 b						
silt		40.0 \pm 0.7 a	66.3 \pm 1.8 b	95.5 \pm 1.3 d	85.4 \pm 1.9 c	85.4 \pm 1.9 c	79.6 \pm 4.2 c	81.8 \pm 0.6 c						
sand		12.3 \pm 1.4 ab	9.1 \pm 1.0 ab	7.0 \pm 0.1 a	9.4 \pm 0.4 ab	9.4 \pm 0.4 ab	15.2 \pm 1.7 b	8.8 \pm 0.6 a						
Ions in 1:5 water extract	mg L ⁻¹	18.5 \pm 0.8 a	33.6 \pm 1.3 a	1529 \pm 48 b	958 \pm 253 b	958 \pm 253 b	1755 \pm 25 b	1176 \pm 185 b						
Cl ⁻		354 \pm 75 d	210 \pm 29 c	36 \pm 5 b	34 \pm 3 b	34 \pm 3 b	8 \pm <1 a	36 \pm 3 b						
SO ₄ ²⁻		5.11 \pm 0.39 d	1.62 \pm 0.08 c	0.75 \pm 0.07 b	0.65 \pm 0.07 b	0.65 \pm 0.07 b	0.15 \pm 0.02 a	1.01 \pm 0.15 b						
Microbial Biomass	mg kg ⁻¹	2.35 \pm 0.18 c	1.23 \pm 0.04 b	0.12 \pm 0.04 a	0.27 \pm 0.06 a	0.27 \pm 0.06 a	< 0.1	1.24 \pm 0.25 b						
Microbial Biomass Carbon (MBC)		56 \pm 3 a	567 \pm 66 b	711 \pm 79 b	481 \pm 42 b	481 \pm 42 b	656 \pm 60 b	698 \pm 36 b						
Dehydrogenase	μ g INTF g ⁻¹ h ⁻¹	< 6	34 \pm 8 a	47 \pm 12 a	34 \pm 2 a	34 \pm 2 a	19 \pm 10 a	23 \pm 8 a						
β -glucosidase	μ mol p-NP g ⁻¹ h ⁻¹	48 \pm 2 a	98 \pm 8 bc	123 \pm 8 c	101 \pm 4 bc	101 \pm 4 bc	93 \pm 5 b	95 \pm 1 bc						
Total metal (loid) concentrations	mg kg ⁻¹	1992 \pm 190 a	9462 \pm 603 b	10460 \pm 180 bc	10100 \pm 541 bc	10100 \pm 541 bc	12342 \pm 616 c	10058 \pm 111 bc						
As		717 \pm 160 a	5866 \pm 275 d	6322 \pm 255 d	4423 \pm 363 c	4423 \pm 363 c	2983 \pm 225 b	2873 \pm 241 b						
Cd		418 \pm 74 a	7730 \pm 491 c	8961 \pm 286 c	8359 \pm 711 c	8359 \pm 711 c	4333 \pm 355 b	4356 \pm 284 b						
Cu		29 \pm 7 a	18 \pm <1 a	14 \pm <1 a	10 \pm <1 a	10 \pm <1 a	19 \pm 2 a	23 \pm 2 a						
Mn		< 10	18 \pm 1 a	16 \pm 3 a	20 \pm 3 a	20 \pm 3 a	< 10	< 10						
Pb		50 \pm 4 b	57 \pm 7 b	13 \pm 3 a	< 10	< 10	17 \pm 8 a	94 \pm 18 b						
Zn		111 \pm 26 a	506 \pm 39 c	464 \pm 156 c	122 \pm 17 ab	122 \pm 17 ab	309 \pm 72 abc	352 \pm 11 bc						
0.01M CaCl ₂ extractable metal (loid) concentrations	μ g kg ⁻¹	< 10	< 10	< 10	< 10	< 10	< 10	< 10						
As		100 \pm 7 a	731 \pm 96 c	753 \pm 102 c	845 \pm 70 c	845 \pm 70 c	173 \pm 17 b	197 \pm 21 b						
Cd														
Cu														
Mn														
Pb														
Zn														

4.3.2. Shannon diversity index for plants and microorganisms

Values of the Shannon diversity index for plant species (Figure 4.3) in plots located at forested sites (*Control* and *External Border*) were higher ($p < 0.05$) than those obtained at the tailings pile, although plots from the *Internal Border* showed intermediate values ($p < 0.05$) between those forest plots and the rest.

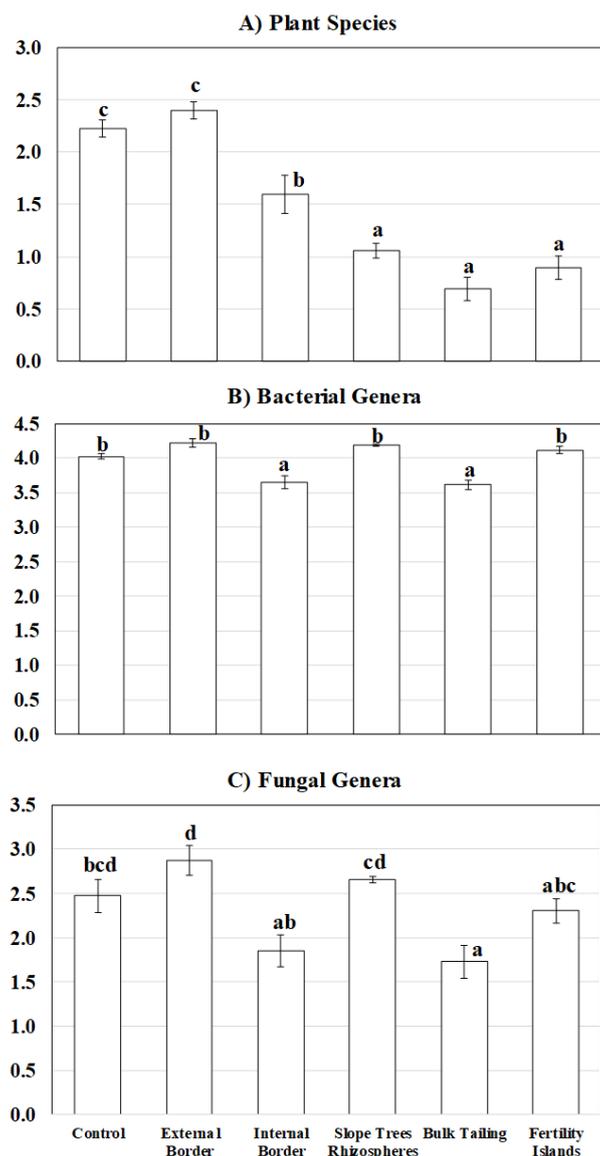


Figure 4.3: Shannon-Weaver index for plants (A), bacteria (B) and fungi (C) at each sampling site. The diversity index for plants corresponds to that obtained in the sampling plots by direct counting, while for bacteria and fungi corresponds to that calculated from abundance data of the soil samples. Bars on columns are standard error. Different letters among columns indicate significant differences (ANOVA with Tukey's test, $p < 0.05$). Number of replicates are 3 for plants and 3 or 6 for bacteria and fungi.

In relation to the bacterial data, the diversity index of non-vegetated soil samples from the tailings pile (*Internal Border* at the slope and *Bulk Tailing* at the plateau) showed lower values ($p < 0.05$) than the rest of samples. For fungal composition, *Bulk Tailing* samples showed the lowest values of diversity index and differed ($p < 0.05$) from forest samples (*Control* and *External Border*) and *Slope Trees Rhizospheres* (Figure 4.3). The highest values of fungal diversity were observed at the *External Border*, which were statistically higher ($p < 0.05$) than those obtained at the plateau (both, *Bulk Tailing* and *Fertility Islands*) and the *Internal Border* (Figure 4.3).

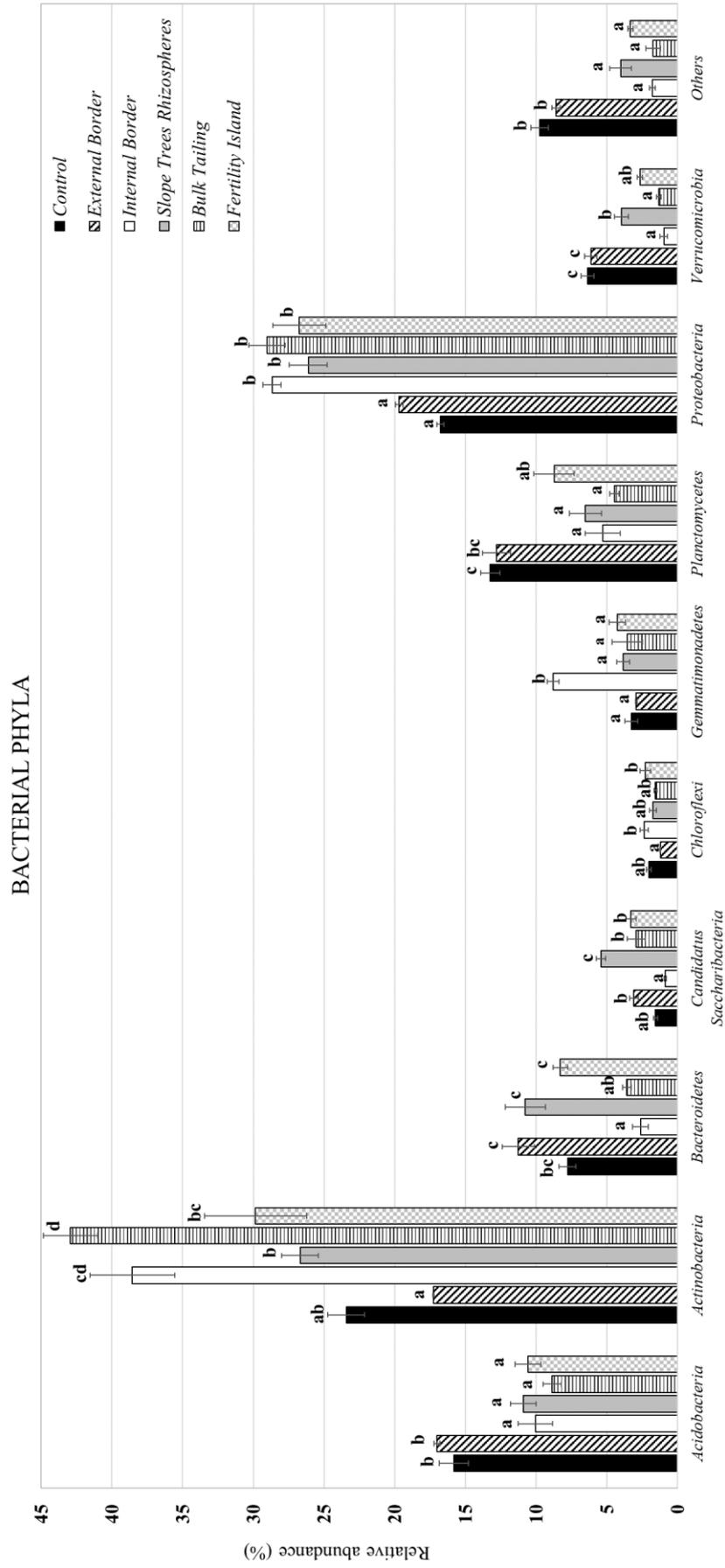
4.3.3. Bacterial and fungal abundance

Analyses of genus taxa were performed for bacteria but most of the genera remained as unclassified (*uncl.* from now on). For this reason, results were based on phyla (Figure 4.4) and orders (Figure 4.6 A and B). The most dominant phyla across all soil samples were *Actinobacteria*, *Proteobacteria* and *Acidobacteria*, which accounted for 50 to 80 % of total bacteria (Figure 4.4).

The *Actinobacteria* phylum showed about 2-fold higher relative abundance in non-vegetated samples ($p < 0.05$, *Bulk Tailing* and *Internal Border*) than in forest samples (*Control* and *External Border*) mainly due to the contribution of three orders belonging to the *Actinobacteria* class: *Acidimicrobiales*, *Actinomycetales* and *Actinobacteria uncl.* (Figure 4.6 A).

In the case of the *Acidobacteria* phylum, all samples from the forest (*Control* and *External Border*) showed significantly higher relative abundance percentages ($p < 0.05$) than the rest of the samples (Figure 4.4) mainly due to the contribution of the *Acidobacteria Gp4* order (Figure 4.6 B).

Figure 4.4: Percentage of bacterial phyla abundance for each sampling site. Bars on columns are standard error. Different letters among columns indicate significant differences (ANOVA with Tukey's test, $p < 0.05$). Number of replicates are 3 or 6.



Percentages of *Proteobacteria* at all the tailings pile samples were higher ($p < 0.05$) than those obtained at forest samples (both, *Control* and *External Border*) (Figure 4.4). Within the *Proteobacteria* phylum, four main bacterial classes were detected: *Alphaproteobacteria*, *Betaproteobacteria*, *Deltaproteobacteria* and *Gammaproteobacteria*. The last class showed the highest differences in abundances between non-vegetated samples at the tailings pile (*Internal Border* and *Bulk Tailing*, $p < 0.05$, Figure 4.5) and the rest of samples, mainly due to the contribution of the *Gammaproteobacteria uncl.* order (Figure 4.6 A). Contrary to this, the *Rhizobiales* order, belonging to the *Alphaproteobacteria* class, showed abundance percentages that were lower by roughly half ($p < 0.05$) in non-vegetated tailings pile samples, relative to abundance percentages in the rest of the samples (Figure 4.6 A).

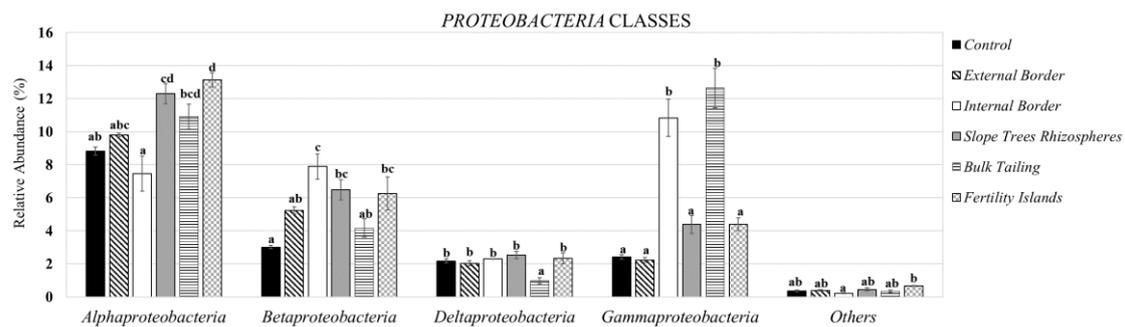


Figure 4.5: Percentage of *Proteobacteria* classes abundance for each sampling site. Bars on columns are standard error. Different letters among columns indicate significant differences (ANOVA with Tukey's test, $p < 0.05$). Number of replicates are 3 or 6.

Samples of vegetated areas at the tailings pile (*Slope Trees Rhizospheres* and *Fertility Islands*) showed higher percentages of *Bacteroidetes* phylum (at least 2-fold, $p < 0.05$, Figure 4.4) than non-vegetated tailings pile samples (*Internal Border* and *Bulk Tailing*), with *Cytophagales* and *Sphingobacteriales* appearing as the most abundant orders (Figure 4.6 B).

Figure 4.6 A: Percentage of bacterial orders abundance for each sampling site. Bars on columns are standard error. Different letters among columns indicate significant differences (ANOVA with Tukey's test, $p < 0.05$). Number of replicates are 3 or 6.

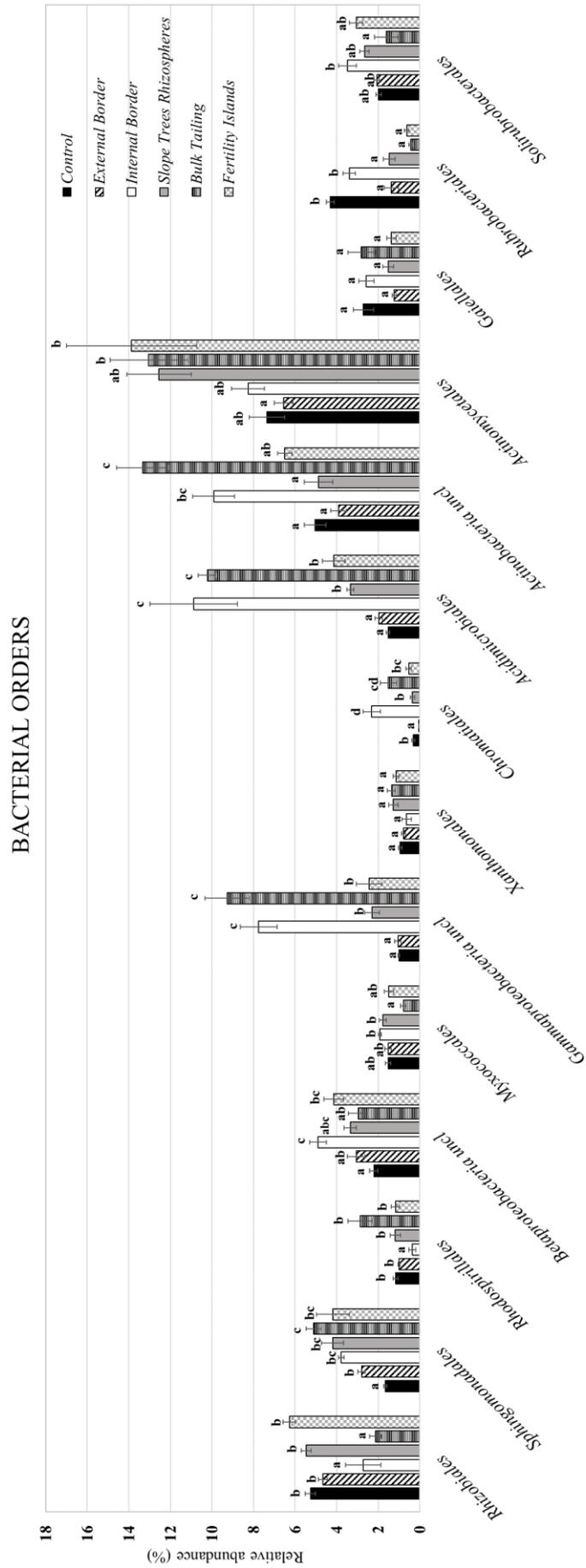
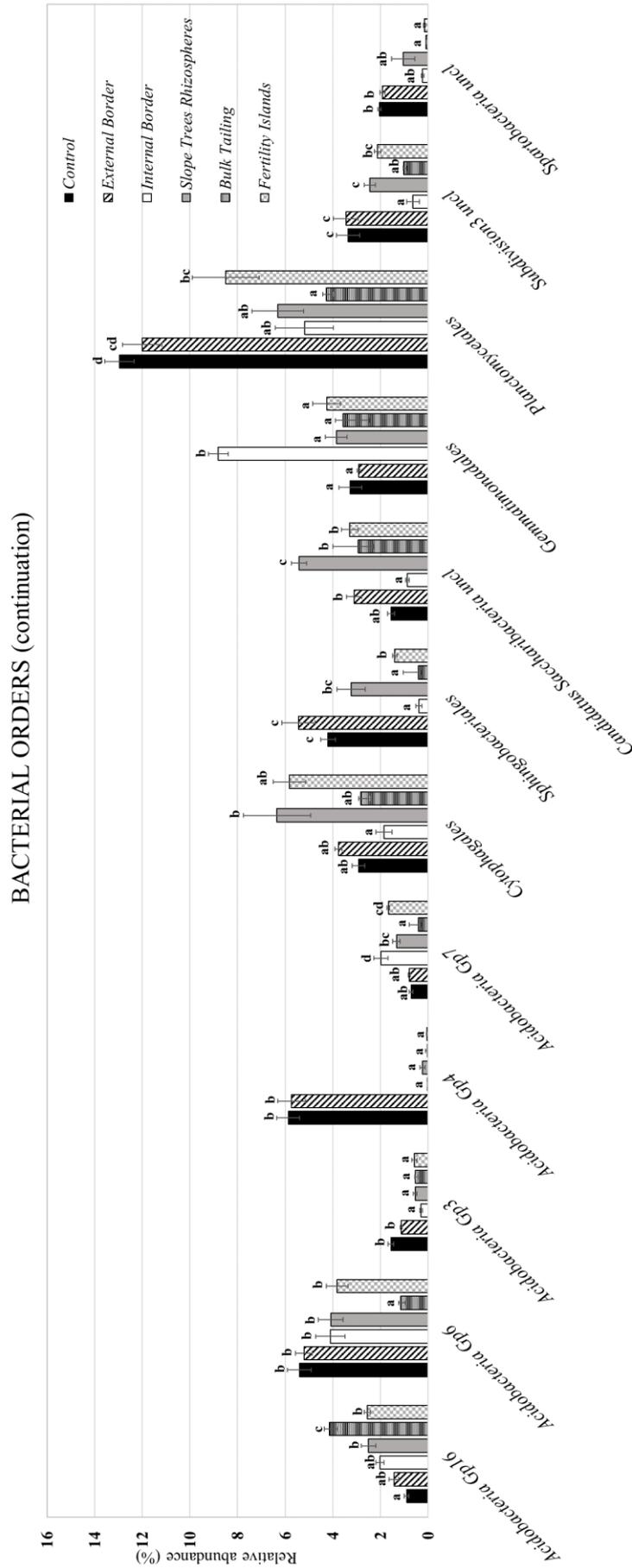


Figure 4.6 B: Percentage of bacterial orders abundance for each sampling site. Bars on columns are standard error. Different letters among columns indicate significant differences (ANOVA with Tukey's test, $p < 0.05$). Number of replicates are 3 or 6.



Samples from the forest (*Control* and *External Border*) showed higher relative abundance percentages ($p < 0.05$) of the *Verrucomicrobia* phylum than all tailings pile samples (Figure 4.4). Similar to this, the *Control* samples showed around 2-fold higher abundance percentages ($p < 0.05$) of the *Planctomycetes* phylum than tailings pile samples. In the case of the *Gemmatimonadales* order (Figure 4.6 B), belonging to *Gemmatimonadetes* phylum, samples from the *Internal Border* showed around 2-fold higher abundance percentages ($p < 0.05$) than the rest of samples. For the *Candidatus Saccharibacteria* phylum (Figure 4.4), only samples from the *Slope Trees Rhizospheres* showed abundances higher than 5 % ($p < 0.05$). The *Chloroflexi* phylum (Figure 4.4) showed abundance percentages below 3 % for all samples, and differences between zones only occurred between the *Internal* and the *External Border*.

Two fungal phyla (Figure 4.7) accounted for more than 90 % abundance: *Ascomycota* and *Basidiomycota*. The *Ascomycota* phylum showed higher abundance percentages (about 2-fold, $p < 0.05$) at the *Control* than at the *External Border* and some tailings pile samples, mainly due to the contribution of the *Ascomytoca uncl.* order (Figure 4.8).

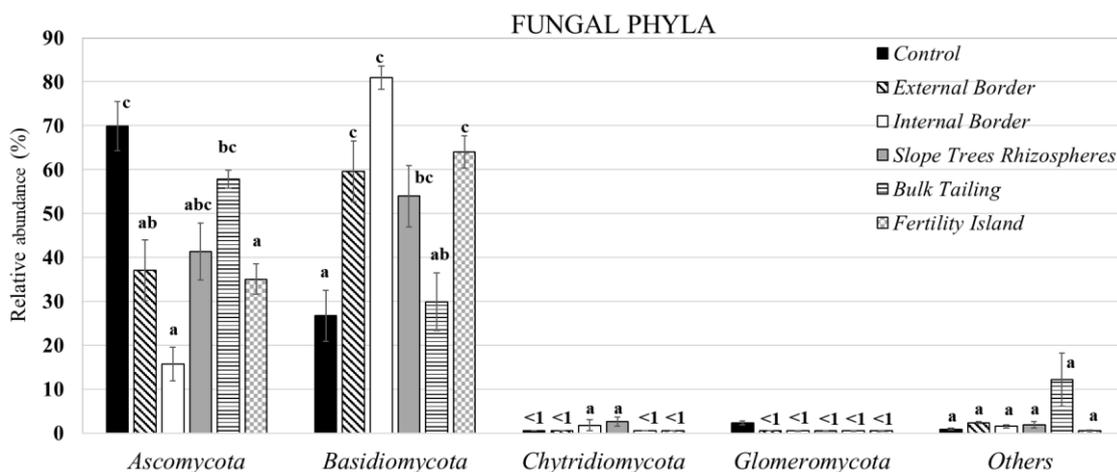


Figure 4.7: Percentage of fungal phyla abundance for each sampling site. Bars on columns are standard error. Different letters among columns indicate significant differences (ANOVA with Tukey's test, $p < 0.05$). Number of replicates are 3 or 6.

The *Basidiomycota* phylum showed higher abundances (about 2-fold, $p < 0.05$) in the borders of the tailings pile (*External* and *Internal*) and in the *Fertility Islands* relative to the *Bulk Tailing* and *Control*, due to the contribution of *Agaricales* and *Thelephorales* orders (Figure 4.8).

The *Glomeromycota* phylum occurred at higher abundance percentages ($p < 0.05$) at *Control* samples, due to the contribution of the *Glomerales* order (Figure 4.8). Finally, there were not differences among zones for the *Chytridiomycota* phylum and *Others* (Figure 4.7).

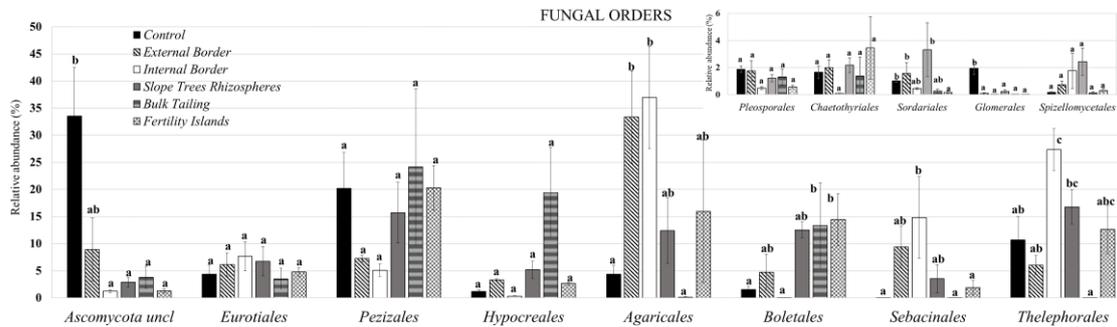


Figure 4.8: Percentage of fungal orders abundance for each sampling site. Bars on columns are standard error. Different letters among columns indicate significant differences (ANOVA with Tukey's test, $p < 0.05$). Number of replicates are 3 or 6.

4.3.4. Soil fertility and its relationship with soil microorganisms

Results of the PCA for soil parameters are shown in the Figure 4.9 and Table 4.3. The two first axes explained 89.9 % of the variance. The PCA1-axis segregated between tailings pile samples (negative side of the axis), defined by adverse soil fertility conditions (e.g. $r < -0.7$ for EC, total Zn or SO_4^{2-w}), and external samples (positive side of the axis) defined by more favourable soil fertility parameters and lower metal concentrations (e.g. $r > 0.9$ for pH, OC, TN or MBC). For the group of tailings pile samples, the PCA2-axis segregated between samples from the tailings' slope (*Internal Border* and *Slope Trees Rhizospheres*, positive side of the axis) and those coming from the plateau (*Bulk Tailing* and *Fertility Islands*, depicted on the negative side). Similarly, for forest samples (*External Border* and *Control*), the PCA2-axis segregated between the *External Border* (positive side) and the *Control* (negative side). The positive PCA2-axis was defined by increasing metal concentrations (e.g. $r > 0.6$ for total Zn and total Pb), while the negative PCA2-axis was conditioned by increasing values of electrical conductivity (e.g. $r \sim -0.5$ for EC and SO_4^{2-w}).

Table 4.3: Results of the Principal Component Analyses (PCA) for selected soil parameters through the transect.

Soil parameters	PCA components	
	PCA 1	PCA 2
pH	0.90	0.37
Electrical Conductivity (EC)	-0.77	-0.52
Organic Carbon (OC)	0.92	0.21
Total Nitrogen (TN)	0.97	0.12
Clay	0.95	-0.17
Sand	-0.96	0.14
CaCl ₂ Extractable Zn	-0.48	0.78
Total Mn	-0.94	-0.02
Total Pb	-0.67	0.66
Total Zn	-0.73	0.64
Water extractable SO ₄ ²⁻	-0.82	-0.47
Microbial Biomass Carbon (MBC)	0.89	0.18
Dehydrogenase	0.96	-0.03

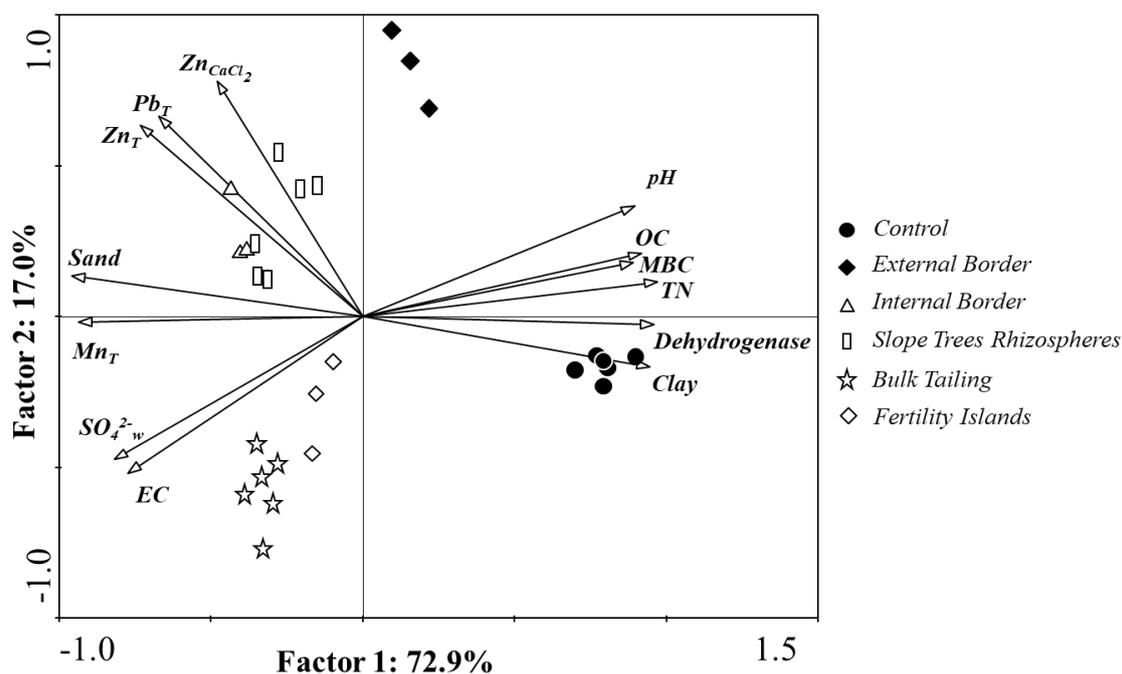


Figure 4.9: Results of the Principal Component Analysis (PCA) for selected soil parameters of the transect samples. “Mt” are total concentrations of the M element; “SO₄²⁻_w” is water extractable sulphate concentrations; “M_{CaCl₂}” are 0.01 M CaCl₂ extractable concentrations of the M element; “OC” is Organic Carbon; “TN” is Total Nitrogen concentrations; “Sand” and “Clay” are the percentages of the corresponding particle size; “EC” and “pH” are Electrical Conductivity and pH, respectively, of the 1:5 soil:water extract; “MBC” is the Microbial Biomass Carbon; Dehydrogenase is the corresponding enzymatic activity.

Results of the CCA for bacterial orders are shown in the Figure 4.10 and Tables 4.4 and 4.5. Analyses were significant (Monte-Carlo test, $p < 0.05$) and the first axis explained 57.4 % of the variance (Table 4.4). The positive side of the CCA1-axis was defined by an improvement of soil fertility parameters (OC, DOC, TN, MBC, dehydrogenase, β -glucosidase, clay, silt) and plant biodiversity (H'_p), while the negative side of the CCA1-axis was conditioned by increasing values of metal concentrations and salinity (EC and SO_4^{2-}). In this way, CCA1-axis segregated between those bacterial orders established in samples from the forest (*Control* and *External Border*) and those developing in non-vegetated tailings` samples (*Bulk Tailing* and *Internal Border*). Vegetated tailings` samples (*Slope Trees Rhizospheres* and *Fertility Islands*) appeared in an intermediate transitional position between the two previously mentioned groups. The CCA1-axis then, indicated a high influence of soil fertility ($r > 0.78$ for pH, OC, DOC, TN, MBC and $r < -0.8$ for EC and water extractable SO_4^{2-} , Table 4.5) and some selected total metal concentrations ($r = -0.7$ for Total As and Mn) on the bacterial orders distribution (Table 4.5). The CCA2-axis (Table 4.5) indicated the influence of plant diversity ($r = -0.45$ for H'_p) and texture factors (e.g. $r = -0.42$ for clay), and differed at tailings pile samples between those from vegetated areas (*Slope Trees Rhizospheres* and *Fertility Islands*) and those from the non-vegetated ones (*Internal Border* and *Bulk Tailing*).

Table 4.4: Data of the Canonical Correspondence Analysis (CCA) including eigenvalues of the two first axis and cumulative percentage of variance of bacteria-soil and fungal-soil data interaction. The significance of the first canonical axis (Monte-Carlo test, p-value) is also provided.

Parameter evaluated	Axis	Eigenvalue	% cumulative variance	Significance
Bacterial orders	I	0.190	57.4	0.008
	II	0.057	74.5	
Fungal orders	I	0.403	28.2	0.362
	II	0.349	52.7	

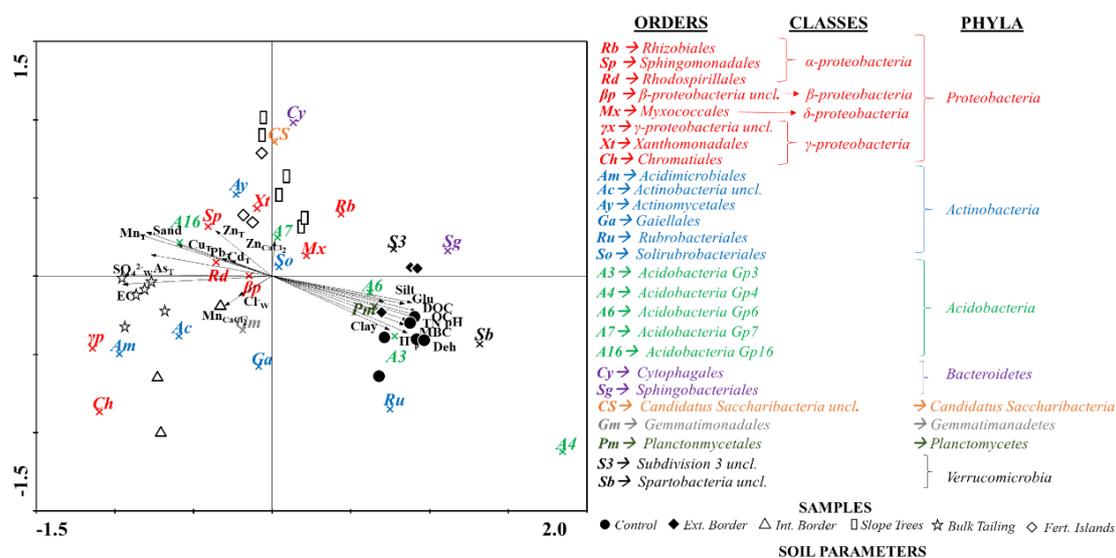


Figure 4.10: Ordination diagram obtained for the Canonical Correspondence Analysis (CCA) of bacterial orders abundance and soil properties. “Mt” are total concentrations of the M element; “SO₄²⁻” and “Cl_w” are water extractable sulphate and chloride concentrations, respectively; “M_{CaCl2}” are 0.01M CaCl₂ extractable concentrations of the M element; “OC” is Organic Carbon; “TN” is Total Nitrogen concentrations; “DOC” is Dissolved Organic Carbon; “Sand” “Silt” and “Clay” are the corresponding percentages of particle size; “EC” and “pH” are Electrical Conductivity and pH, respectively, of the 1:5 soil:water extract; “MBC” is the Microbial Biomass Carbon; Deh and Glu are the corresponding dehydrogenase and β-glucosidase enzymatic activities; “H_p” is the Shannon diversity index for plants.

Results of the CCA for fungal orders are shown in the Figure 4.11 and Tables 4.4 and 4.5. Contrary to what occurred for bacteria, for fungal orders analyses were not significant and the first axis explained 28.2 % of the variance. The accumulated percentage for variance explanation of two first axes was of 52.7 % (Table 4.4). The positive side of the CCA1-axis was defined by increasing values of Cl_w, while the negative side was mainly conditioned by increasing values of metal concentrations. In this way, CCA1-axis segregated *Bulk Tailing* samples from the rest. The positive side of the CC2-axis was defined by increasing values of soil fertility parameters, while the negative was done by metals and salinity. The CCA1-axis showed Cl_w (r=0.71) and metals (e.g. r=-0.66 Pb) as main parameters for fungal orders segregation (Figure 4.11 and Table 4.5). The CCA2-axis showed a high influence of soil fertility parameters (e.g. r=0.71 for TN, r=-0.64 for EC) and plant biodiversity (r=0.77 for H_p) in the segregation of some *Control* samples from the rest of samples (Figure 4.11 and Table 4.5).

Table 4.5: Weighted correlation matrix for the first two species axes and environmental variables for bacterial and fungal orders.

Soil parameter	Bacterial orders		Fungal orders	
	Species Ax1	Species Ax2	Species Ax1	Species Ax2
pH	0.87	-0.32	-0.04	0.76
Electrical Conductivity (EC)	-0.88	-0.06	0.12	-0.64
Organic Carbon (OC)	0.85	-0.28	0.07	0.59
Dissolved Organic Carbon (DOC)	0.78	-0.22	0.05	0.50
Total Nitrogen (TN)	0.84	-0.33	0.14	0.71
Particle size distribution	Clay	0.69	-0.42	0.32
	Silt	0.66	-0.20	0.39
	Sand	-0.74	0.35	-0.39
CaCl ₂ extractable metals	Mn	-0.28	-0.23	-0.38
	Zn	0.01	0.28	-0.58
Total Metal(loid) concentration	As	-0.71	0.17	-0.30
	Cd	-0.25	0.12	-0.51
	Cu	-0.56	0.25	-0.55
	Mn	-0.78	0.33	-0.14
	Pb	-0.30	0.14	-0.66
	Zn	-0.33	0.36	-0.64
Water Extractable ions	Cl ⁻	-0.20	-0.15	0.71
	SO ₄ ²⁻	-0.88	0.01	0.13
Microbial Biomass Carbon (MBC)	0.80	-0.35	0.14	0.69
Enzymatic activities	dehydrogenase	0.78	-0.38	0.21
	β-glucosidase	0.82	-0.21	0.16
Shannon diversity index for plant species (H')	0.80	-0.45	-0.20	0.77

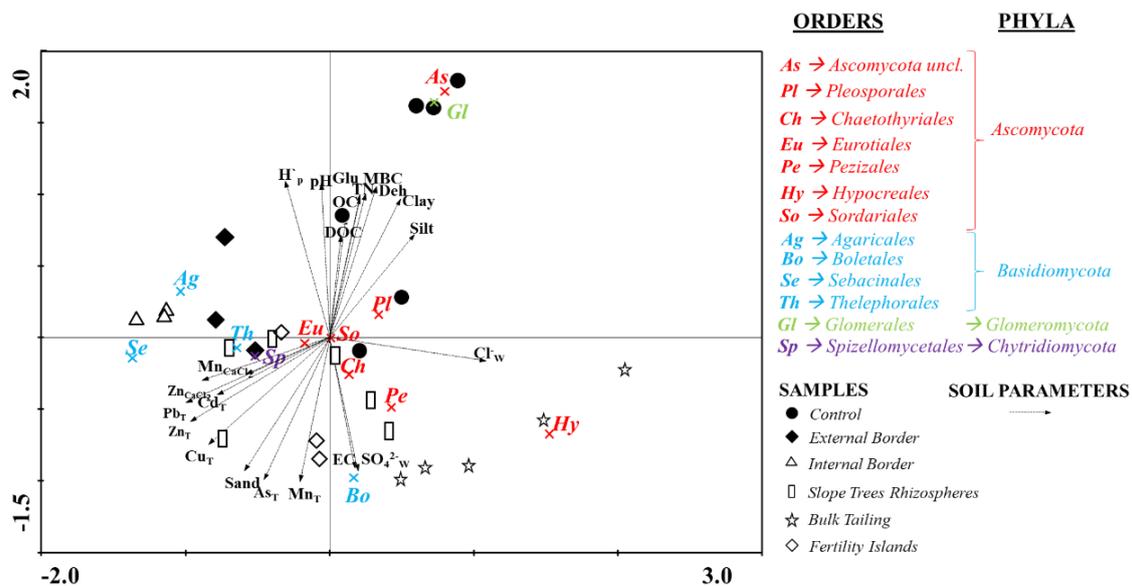


Figure 4.11: Ordination diagram obtained for the Canonical Correspondence Analysis (CCA) of fungal orders abundance and soil properties. “Mt” are total concentrations of the M element; “SO₄^{2-w}” and “Cl⁻” are water extractable sulphate and chloride concentrations, respectively; “M_{CaCl₂}” are 0.01M CaCl₂ extractable concentrations of the M element; “OC” is Organic Carbon; “TN” is Total Nitrogen concentrations; “DOC” is Dissolved Organic Carbon; “Sand”, “Silt” and “Clay” are the corresponding percentages of particle size; “EC” and “pH” are Electrical Conductivity and pH, respectively, of the 1:5 soil:water extract; “MBC” is the Microbial Biomass Carbon; Deh and Glu are the corresponding dehydrogenase and β-glucosidase enzymatic activities; “H’_p” is the Shannon diversity index for plants.

4.4. Discussion

4.4.1. Environmental niches associated with the experimental transect

The variation in soil properties through the experimental sampling transect were mainly driven by parameters such as pH, OC, DOC or TN, which correlated positively with MBC and soil enzymatic activities ($r > 0.67$, $p < 0.01$) and negatively with total metal(loid) concentrations (e.g. $r < -0.7$, $p < 0.01$ for Mn) and EC ($r < -0.7$, $p < 0.01$). In this way, *Control* samples showed the most favourable soil fertility parameters (e.g. low EC, high OC, DOC, TN, MBC) and lower metal load, while *Bulk Tailing* samples reflected the most adverse conditions for plant growth (higher EC, low OC, DOC, TN and MBC) under a similar metal load to the rest of tailings pile samples. As previous research on mine tailings has shown, the variability in total metal concentrations among different tailings pile's sites under similar pH conditions may not be the single cause for generating specific niches for plant/microbial development, being other additional physico-chemical factors such as salinity or texture important for explaining the occurrence of ecological successional processes (Anawar et al., 2013; Colin et al., 2019). The main contributor to salinity at our tailings pile came from SO_4^{2-} . Its concentration showed a tight correlation with EC ($r = 0.93$, $p < 0.01$). The high concentration of SO_4^{2-} in samples that determined EC ($r = 0.98$, $p < 0.01$), was not attributable to acidifying reactions but to the generation of secondary minerals such as gypsum, which did not cause a decrease in pH (Párraga-Aguado et al., 2013). In relation to metals distribution at the tailings pile, slopes were enriched in Pb and Zn, while plateau showed higher Mn concentrations. These differences were attributed to the inherent heterogeneity of bulk materials of tailings and not to a secondary redistribution of metals. In spite of higher metal concentrations (e.g. total and CaCl_2 extractable for Zn) and similar ranges of EC to those obtained for some sites of the plateau, the *Internal Border* showed higher plant biodiversity than the rest of tailings pile's sites (Figure 4.3) probably due to the proximity of the forest and higher probability of receiving seeds.

4.4.2. Importance of the shifts of bacterial composition in the edaphic gradient

The description of changes in bacterial abundances at phylum level could serve as a general approach to identify the most relevant effects of environmental stressors (e.g. metals, salinity) on bacterial compositions in polluted sites (e.g. Wakelin et al., 2012; Pepper and Gentry, 2015; Rosenfeld et al., 2018; Sun et al., 2018).

At our samples, three bacterial phyla (*Actinobacteria*, *Proteobacteria* and *Acidobacteria*) accounted for 50 to 80 % of all bacteria (Figure 4.4). *Proteobacteria* and *Actinobacteria* were the most abundant phyla in tailings pile samples, accounting for more than 50 % of the total bacterial abundance, which is in accordance to previous findings, which showed that high concentrations of metal(loid)s stimulate competitiveness (e.g. Gremion et al., 2003; Narendrula-Kotha and Nkongolo, 2017; Rosenfeld et al., 2018; Sun et al., 2018). The *Acidobacteria* phylum, the third most abundant across all samples, is known to be widely distributed in extreme environments such as strongly acidic tailing wastes, where it appears as the most abundant phylum (e.g. Wakelin et al., 2012; Kielak et al., 2016; Sun et al., 2019), showing a high dependence of this bacteria on soil pH (Jones et al., 2009). Thus, in our study, the neutral pH of the tailings substrate may have prevented the occurrence of this phylum at higher abundance percentages.

The evaluation of more specific taxa (e.g. orders, genera) could be more suitable for revealing aspects related to bacterial functionality or edaphic succession. For this purpose, we focus on the analysis of Figure 4.10, in which it is possible to observe those bacterial orders, which are associated with each sampling site. For the interpretation of the CCA Figure, it is important to state that the discussion will be focused on differences among sampling sites and not in the absolute abundance of each order.

Variations in bacterial abundance were better explained by soil fertility related factors, such as organic matter (OC, DOC, TN), pH, salinity (EC), particle size percentages, enzymatic activities (dehydrogenase and β -glucosidase) or microbial biomass than by metal concentrations. Only some specific total metal concentrations, such as As or Mn, showed a strong influence on

the CCA1-axis (Figure 4.10 and Table 4.5). Three groups of samples were generated in Figure 4.10: a first group formed by non-vegetated samples at the tailings pile; a second group constituted by vegetated samples from the tailings pile; and finally, a third group formed by samples taken out outside of the tailings pile (forest sites). While samples from the forested zones (*Control* and *External Border*) were grouped nearly together, samples from the tailings pile were not grouped according to their spatial situation (e.g. *Fertility Islands* did not group together with the *Bulk Tailing* of the plateau) but whether or not there was vegetation present.

The first cluster of samples was placed at the negative side of the CCA1-axis (opposite to *Control* and *External Border* samples, Figure 4.10) and was composed of non-vegetated samples of the tailings pile (the *Bulk Tailing* and marginally, the *Internal Border*). These samples were characterized by adverse soil fertility conditions (low OC, DOC and TN, low MBC and enzymatic activities, high electrical conductivity) and high metal(loid) concentrations. These edaphic characteristics might provide conditions for the occurrence of microbial colonizers in the early successional stages (Colin et al., 2019). Orders which showed higher abundances in these sites include *Gammaproteobacteria*'s orders such as *Gammaproteobacteria uncl.* and *Chromatiales*, and *Actinobacteria*'s orders such as *Acidimicrobiales* and *Actinobacteria uncl.* (Figures 4.10 and 4.6 A). As it was explained above, *Proteobacteria* and *Actinobacteria* phyla are known to contain specific taxa with dominance in barren metal(loid) enriched materials because of their lithotrophic nature (Wakelin et al., 2012). Abundances of those *Gammaproteobacteria*'s orders showed a strong positive correlation with SO_4^{2-} concentration ($r > 0.6$, $p < 0.01$). This correlation is supported by the well-known relation of the *Chromatiales* taxa with the sulphur cycle (Imhoff, 2005). Similarly, it is known that there is an affinity for enriched sulphur environments of the *Acidimicrobiales* order, belonging to the *Actinobacteria* phylum (Sun et al., 2018).

Several orders appeared in an intermediate position, sharing similar abundances between the slope and plateau samples (Figure 4.6 A, B and 4.10). These bacterial orders included, among others, an order of *Acidobacteria* and three orders of *Proteobacteria*: *Betaproteobacteria uncl.*,

Rhodospirales and *Sphingomonadales*. The last order was mainly represented by the *Sphingomonas* genus (Figure 4.12), which has been described previously in polluted environments (Baraniecki et al., 2002; Wang et al., 2019).

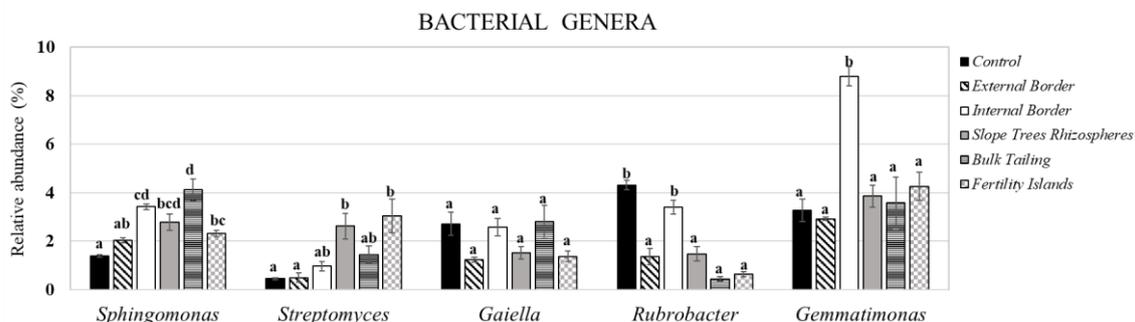


Figure 4.12: Percentage of identified bacterial genera abundance for each sampling site. Bars on columns are standard error. Different letters among columns indicate significant differences (ANOVA with Tukey's test, $p < 0.05$). Number of replicates are 3 or 6.

The second group of samples was formed by vegetated tailings pile samples and appeared in the positive side of the CCA2-axis, showing high metal(loid) concentrations and intermediate values of soil fertility parameters between forest samples (*Control* and *External Border*) and non-vegetated tailings pile samples (Figure 4.10). These samples were characterized by higher abundances of *Cytophagales* (belonging to the *Bacteroidetes* phylum), *Candidatus Saccharibacteria uncl.* (belonging to the *Candidatus Saccharibacteria* phylum) or *Actinomycetales* (belonging to the *Actinobacteria* phylum) orders (Figure 4.6 A and B). The increase in bacterial diversity indices in these vegetated samples of tailings in relation to non-vegetated samples (Figure 4.3) could be related to the improvement in soil fertility parameters (e.g. OC, DOC), coming from rhizosphere mediated processes (exudates, respiration) and the generation of litter (Pugnaire et al., 2011; Colin et al., 2019). These conditions may reconfigure the structure of former bacterial communities of bare areas of tailings, adapted to adverse soil conditions and with lithotrophic characteristics, to communities more organotrophic, in which new nutrient resources may open new ecological niches (Huang et al., 2011). In addition, the presence of more nutrient resources may prevent growth of low-competitive bacteria for growth (Uksa et al., 2015). This is clearly seen in our data, where lithotrophic orders of

Gammaproteobacteria (e.g. *Gammaproteobacteria uncl.*) and *Actinobacteria* (*Acidimicrobiales*), which dominated at non-vegetated tailings pile samples, showed a significant lower abundance in vegetated patches of the tailings pile (Figure 4.6 A and 4.10). By contrast, vegetated samples at the tailings pile showed a higher enrichment in the abundance of organotrophic bacteria such as the *Cytophagales* order (*Bacteroidetes* phylum), which has been shown to play an important role in the decomposition of organic matter under neutral pH environments (Reichenbach, 2006), or the *Actinomycetales* order, which has been related to plant rhizospheres, stimulating organic matter decomposition, atmospheric N fixation and phosphate availability (Hozzein et al., 2019). Within this order, the *Streptomyces* genus (which showed higher abundance in vegetated tailings samples, Figure 4.12) is able to stimulate the mycorrhizal growth and therefore improve plant establishment (Schrey et al., 2012). The *Candidatus Saccharibacteria* order, which had its highest abundance at the *Slope Trees Rhizosphere*, has also been related to soil metals and specifically associated to plant rhizospheres at mine tailings (Navarro-Noya et al., 2010; Ni et al., 2016). Thus, the specific type of plant species, which act as nutrient suppliers may modulate bacterial compositions by generating different types root exudates, litter, etc (Fierer et al., 2010). We may expect that vegetated areas of the tailings pile, which showed a similar composition of plant species to the *External Border* (see Table 4.1), could share some bacteria groups. This was the case of *Rhizobiales* (*Alphaproteobacteria* class), *Sphingobacteriales* (*Bacteroidetes* phylum), *Planctomycetales* (*Planctomycetes* phylum) and *Subdivision 3 uncl.* (*Verrucomicrobia* phylum) orders (Figure 4.10). In the specific case of the *Rhizobiales* order, similar abundances were observed in vegetated tailings and forest samples (including the *Control*) (Figure 4.6 A). It is known that this order includes nitrogen fixers and, in the case of being present in barren materials, its occurrence is related to the establishment of vegetation (Jones, 2015). Several studies have revealed the importance of *Rhizobiales* in the enhancement of plant resistance to high soil metal(loid) concentrations (Chen et al., 2008; Hao et al., 2014). The other three shared orders among vegetated tailings and forest samples have been described as belonging to generalist phyla with role in the C cycle, such as the case of the *Sphingobacteriales* order (*Bacteroidetes* phylum) (Hester et al., 2018).

Those shared orders would link vegetated tailings samples to the last third group of samples that were placed at the positive side of CCA1-axis and were composed by samples of *Control* and *External Border* sites (Figure 4.10). *Control* samples were characterised by better soil fertility conditions (higher clay percentages, OC and TN concentrations and soil enzymatic activities) and lower metal(loid) concentrations, which may constitute conditions for the mature step in the edaphic succession. In relation to the *Control* site, *External Border* samples shared similar values for fertility parameters but significantly higher metal(loid) concentrations. Orders identified in both forested sites included aforementioned orders, which were shared with vegetated tailings samples (Figures 4.6 A, B and 4.10). Other additional orders belong to *Verrucomicrobia* (*Spartobacteria uncl.*), *Acidobacteria* (*Acidobacteria Gp3*, *Gp4* and *Gp6*) and *Actinobacteria* (*Rubrobacteriales*) phyla. Most of these orders showed similar abundances ($p < 0.05$, Figure 4.6 A and B) between *Control* and *External Border* samples. Taking a look at the rest of orders identified through the transect (Figure 4.6 A and B), only the *Chromatiales* order showed differences between these two zones. This similarity between bacterial compositions of *Control* and *External Border* sites may give an interesting insight in relation to the effect of high metal(loid) concentrations on bacterial composition. Although metal(loid)s toxicity in the *External Border* may have affected microbial diversity and/or its structure, tolerant/adapted microorganisms may proliferate under long-term periods, even under high metal(loid) loads, and support the occurrence of values of diversity indexes similar to those obtained at the previous non-impacted conditions (Pereira et al., 2014). In addition, the presence of organic matter (OC, DOC) at similar values to the *Control* site may have contributed to a diminished toxicity of metals, acting as a buffer, and contributing to the microbial survival (Pennanen, 2001). This may explain the occurrence of similar microbial diversity indices and abundances for most of the orders, when comparing the *External Border* and *Control* sites, in spite of showing contrasting metal(loid) concentrations (e.g. 10-fold As or 8-fold Pb higher at the *External Border*). However, the impact of high metal(loid) concentrations at the *External Border* may have affected microbial activity as it was shown by the lower MBC and enzymatic activities (Kandeler et al., 2000). This behaviour showed that, under adequate nutrient supply and optimum pH, bacterial communities may show

better adaptation to environmental stressors such as high metal(loid) concentrations. The *External Border*, with its similar metal(loid) load to the tailings pile, could serve as a “model” to mimic the restoration of the tailings. In fact, the study of transitional areas (those surrounding areas to mine tailings piles such as the case of the *External Border*) may provide an interesting approach to adaptation mechanisms of site-specific tolerant microorganisms, due to being partially influenced by the characteristics of mine tailings (Takeuchi and Shimano, 2009; Anawar et al., 2013).

4.4.3. Importance of the shifts of fungal composition in the edaphic gradient

Fungal communities at tailings were dominated by *Ascomycota* and *Basidiomycota* phyla. Similar results were observed in other metals polluted sites (e.g. Op De Beeck et al., 2015; Narendrula-Kotha and Nkongolo, 2017; Rosenfeld et al., 2018). Both groups (*Ascomycota* and *Basidiomycota*) are by far the most studied fungi phyla. *Ascomycota* shows a wide range of lifestyles such as saprotrophs, necrotrophic or biotrophic parasites of plants and animals, with some existing as endophytes in symptomless associations with plants (Webster and Weber, 2007). On the other hand, *Basidiomycota* may play an important role for decomposing litter and wood and might include ectomycorrhizal fungi, forming mutualistic associations (Webster and Weber, 2007). Abundances of both phyla showed a strong negative correlation through the transect ($r=-0.95$, $p<0.01$), which may indicate a strong competition between both phyla (Egidi et al., 2019) or substitution due to their selective preference by specific/different organic matter substrates (Ma et al., 2013).

Unlike bacteria, variations in fungal abundances through the transect were not so clearly explained by soil parameters (CCA1-axis explained lower percentage of variance than for bacteria). This could have been caused by a lower sensitivity of fungal diversity to environmental stressors such as drought or salinity (Moynahan et al., 2002; Op De Beeck et al., 2015; Thiem et al., 2018). Interestingly, the distribution of samples followed a spatial pattern (samples of consecutive steps of the transect were depicted together), which might suggest different

mechanisms of migration/establishment than bacteria (Moynahan et al., 2002). In relation to fungal diversity (Table 4.3), there was higher variability than for bacteria occurring between vegetated sampling sites of the tailings pile and forest samples, revealing the importance of the specificity of fungi-plant species associations for the fungal composition (Op De Beeck et al., 2015).

In our study, only a few samples of the *Control* area, the *Internal Border* and *Bulk Tailing* samples seemed to differentiate from the rest of samples (Figure 4.11). Samples from the *Control* area showed higher abundance of *Ascomycota uncl.* (*Ascomycota* phylum) and *Glomerales* (*Glomeromycota* phylum) orders. The last order has been intensively studied as it contains the *Glomus* genus, which is a very important group of arbuscular mycorrhizal fungi (AMF) (Gałazka and Grzadziel, 2018). *Bulk Tailing* samples appeared associated to the *Hypocreales* order (*Ascomycota* phylum) and were characterised by increasing Cl_w concentrations (see CCA1-axis in Figure 4.11 and Table 4.5). These results may corroborate the findings of Bastida et al. (2017), which associated the *Hypocreales* order with resistance to abiotic stresses as drought. The highest affinity of the *Basidiomycota*'s orders, such as *Agaricales*, *Sebacinales* and *Thelephorales* for *Internal Border* samples (and marginally for External Border samples) could be related more to the aforementioned antagonism with respect to *Ascomycota* (competition for substrate) or plant specific dependence than to the effect of edaphic factors.

4.5. Conclusions

The study of specific microenvironments at a mine tailings impacted site revealed a contrasting effect of soil properties and presence of vegetation on bacterial and fungal composition and in turn on soil functionality. While bacterial composition was more influenced by edaphic factors, which in turn was related to the presence of plants, fungal composition seemed to be more dependent on spatial distribution or the type of organic substrate.

The presence of plants in vegetated patches at tailings revealed a positive effect on soil fertility and microbiological indicators, stimulating the presence of bacterial orders involved in

biogeochemical cycles. This functionality of the impacted sites, which even showed unfavourable edaphic conditions, may support its long-term sustainability. As a consequence, the phytomanagement of tailings could be considered a suitable technique to achieve their long-term stabilization by the synergistic interaction between native microbial communities and spontaneous vegetation. Further research should focus on evaluating the influence of specific plant species combinations to favour those relevant bacterial and fungal taxa, which play a key role in biogeochemical cycles.

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**CHAPTER 5. Edaphic factors determining the colonization of
semiarid mine tailings by a ruderal shrub and two tree plant
species: implications for phytomanagement**

Edaphic factors determining the colonization of semiarid mine tailings by a ruderal shrub and two tree plant species: implications for phytomanagement

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Abstract

Phytomanagement has been considered a feasible technique to decrease the environmental risks associated to mine tailings and its implementation relies on a suitable plant species selection. The goal of this study was to identify the edaphic factors, including microbiology, affecting the establishment of plant species with contrasting growth patterns during the phytomanagement of mine tailings. For this purpose, a comprehensive rhizosphere characterization was performed in an early ruderal colonizer, *Zygophyllum fabago* and two late successional tree species, *Pinus halepensis* and *Tetraclinis articulata*, growing at a mine tailings pile in southeast Spain. The neutral pH of the tailings determined low 0.01 M CaCl₂ metal extractable concentrations (e.g. <10 µg kg⁻¹ Pb and Cd). Thus, other soil properties different from metal concentrations resulted more determining to explain plant establishment. Results revealed that *Z. fabago* selectively colonized tailings patches characterized by high salinity (3.5 dS m⁻¹) and high silt percentages (42 %), showing a specific halotolerant rhizospheric microbial composition, such as the bacterial *Sphingomonadales* and *Cytophagales* orders and the fungal *Pleosporales* and *Hypocreales* orders. The two tree species grew at moderate salinity areas of the tailings pile (1.7 dS m⁻¹) with high sand percentages (85 %), where *Actinomycetales* was the most abundant bacterial order (>10 % abundance). The contrasting mycorrhizal behaviour of both tree species (ectomycorrhizal for *P. halepensis* and endomycorrhizal for *T. articulata*) could explain the differences found between their fungal rhizospheric composition. In terms of phytomanagement, the selective plant species colonization following specific soil patches at mine tailings would increase their biodiversity and resilience against environmental stressors.

5.1. Introduction

Mine tailings piles are considered a critical environmental issue at former metal sulphide mine sites (Mendez and Maier, 2008; Conesa and Schulin, 2010). These materials come from the left-over of metal refining works and, when uncovered, can cause environmental health concerns because of acting as potential sources of metal(loid) enriched particles (Conesa and Schulin, 2010). The phytomanagement in terms of phytostabilization consists of using plants to prevent water/wind erosion and has been considered as a suitable technique to decrease the environmental risks associated to mine tailings (Robinson et al., 2009). Previous works have revealed that plant choice is a critical step to achieve the successful revegetation in these systems because plants must cope with combined edaphic stress factors such as high metal(loid) concentrations, low fertility, extreme pHs or high salinity (Párraga-Aguado et al., 2013). In addition, recent research has revealed the importance of ecological plant assemblage or species complementarities to maximize the scarcity of nutrient resources at mine tailings (Colin et al., 2019). For instance, prime tailings colonizers are normally composed by fast growth plant species of opportunist and ruderal behaviour, which easily spread in absence of competitors. This pioneer vegetation may play an important role in stimulating plant growth promoting bacteria and providing primary sources of organic matter in these systems (Párraga-Aguado et al., 2014b). Among this pioneer vegetation, the *Zygophyllaceae* *Zygophyllum fabago* L. has been highlighted due to its remarkable environmental plasticity to cope with simultaneous abiotic stress factors in semiarid areas (Párraga-Aguado et al., 2016). Other similar species in these semiarid areas may include *Gramineae* such as *Piptatherum miliaceum* (L.) Cosson, *Chenopodiaceae* such as *Atriplex halimus* L., or *Compositae* such as *Dittrichia viscosa* (L.) Greuter (Párraga-Aguado et al., 2014a). On the other hand, late successional species, normally composed by trees, may promote the occurrence of rhizosphere related microorganisms specialized in the sustainment of nutrient cycles (Colin et al., 2019). For this reason, compared to ruderal species, trees may trigger remarkable feedbacks on the long-term sustainability of the system (e.g. by canopy effect) (Párraga-Aguado et al., 2014b).

Recent research in the phytomanagement of mine tailings have pointed out the importance of soil microbiology for the establishment of a self-sustainable vegetation (Kolaříková et al., 2017; Sun et al., 2018). In turn, soil microbial composition is highly dependent on the specific rhizosphere microbiome. This plant species-microbial community dependence is influenced by root exudates or plant litter, which differ in their composition among plant species (Deng et al., 2018; Sun et al., 2018). Then, an important question in these systems is to determine the relationship between edaphic parameters and the specific rhizosphere microbiome during the plant establishment at mine tailings. In this sense, the comparison between fast growth ruderal colonizers and slow growth tree species could provide an important key to elucidate the role that vegetation plays during the phytomanagement of mine tailings.

The goal of this study was to identify the edaphic factors, including microbiology, affecting the establishment of plant species with contrasting growth patterns during the phytomanagement of a mine tailings pile. For this purpose, we analysed soil parameters and microbial composition in the rhizospheres of the ruderal colonizer *Zygophyllum fabago* and two late successional tree species, *Pinus halepensis* and *Tetraclinis articulata*, growing at a mine tailings pile and at a control site from the semiarid Cartagena-La Unión mining district (southeast Spain).

5.2. Material and Methods

5.2.1. Study site

Sampling site was located at a former mine disposal tailings pile from the semiarid Cartagena-La Unión Mining District (southeast of Spain, 0-385 m a.s.l.; 37° 37' N, 0° 49' W– 37° 35' N, 0° 50' W, ~50 km², Figure 5.1). Annual rainfall accounts for around 250-300 mm yearly, while annual average temperature is 18 °C. Mining activity was based on metallic sulphur minerals such as galena, pyrite or sphalerite. More information on the environmental impacts of the mining activity at this area is available in Conesa and Schulin (2010).

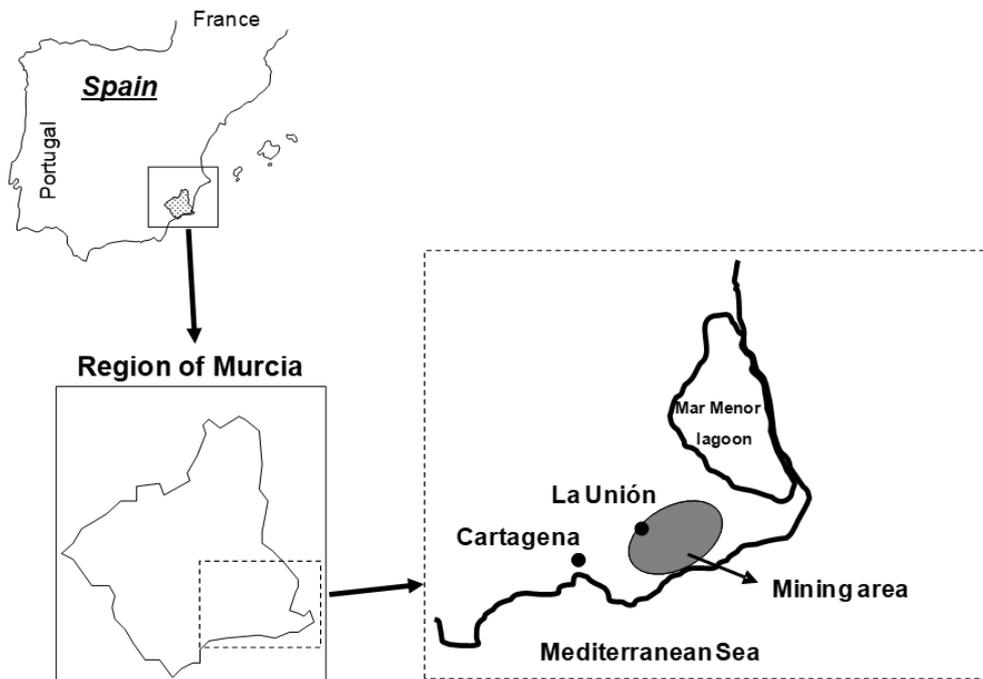


Figure 5.1: Location of the studied area.

5.2.2. Selected plant species and sampling

Three plant species, which spontaneously grow at the mine tailings pile, were chosen: 1) *Pinus halepensis* Miller (Figure 5.2), is an obligate mycorrhizal tree (Querejeta et al., 1998) proposed as a suitable plant species for the restoration of degraded lands in semiarid areas (Querejeta et al., 2008) including metal enriched tailings (Párraga-Aguado et al., 2014b); 2) *Tetraclinis articulata* (Vahl) Mast. (Figure 5.2), belonging to the *Cupressaceae* family, is an evergreen coniferous tree included in the red lists of threatened species (Sánchez Gómez et al., 2011); and 3) *Zygophyllum fabago* L. (Figure 5.2) is an halophyte plant species, which has shown high environmental plasticity for combined abiotic stress factors (Párraga-Aguado et al., 2016). The selected trees are considered late successional species of the Mediterranean forests in southern Spain (Párraga-Aguado et al., 2014b).

*Pinus halepensis**Tetraclinis articulata**Zygophyllum fabago*

Figure 5.2: Images of the selected plant species growing at the sampled tailings pile.

In this study, soil samples from the rhizospheres of the three selected plant species were taken from two locations: plants growing at a mine tailings pile and plants growing at a control site. The latter consists of an open forest located 2 km away from the mine tailings pile. In addition, soil from bare areas at the mine tailings was sampled too. Three composite replicates, each one formed by three sub-samples, were obtained for each zone and plant species. Rhizosphere samples consisted of the volume of soil surrounding few centimetres from the root epidermis (Samad et al., 2019). It was not possible to take bare soil samples at the control site due to its dense vegetation cover. An aliquot of each composite sample was stored in sterile falcon tubes, kept in ice for transportation into the laboratory, sieved through a 2 mm screen and stored at -20°C till enzymatic and microbial analysis were performed. Soil samples for edaphic characterization were air dried at room temperature, sieved through 2 mm, homogenised and stored in sealed plastic bags prior to lab analytical procedures.

5.2.3. Soil Analyses

The pH and Electrical Conductivity (EC) were measured in 1:5 soil:water ratio extracts. These extracts were also used for measuring water extractable anions (Cl^- and SO_4^{2-}) and Dissolved Organic Carbon (DOC) by employing an Ion Chromatographer (Metrohm) and a TOC-automatic analyser (TOC-VCSH Shimadzu), respectively. Metals extractability was assessed by a 1:2.5 soil:0.01 M CaCl_2 solution extraction (González et al., 2011). The resulting extracts were analysed using an ICP-MS (Agilent 7500A) for metals (Cd, Cu, Mn, Pb, Zn). Total Nitrogen (TN)

was determined following the Kjeldahl method and particle size distribution according to the method of Bouyoucos` densimeter. Organic Carbon (OC) concentrations were obtained after oxidising organic matter with potassium dichromate (Duchaufour, 1970). Total element concentrations were determined in ground sub-samples by X-ray fluorescence (Bruker S4 Pioneer).

The soil enzymatic activities dehydrogenase (García et al., 1993) and β -glucosidase (Reboreda and Caçador, 2008) were measured employing unaltered portions of soil samples stored at -20°C. Microbial Biomass Carbon (MBC) was determined by the measurement of the extractable organic carbon by 0.5 M K₂SO₄ after a 24 h CHCl₃-fumigation (Vance et al., 1987; Wu et al., 1990) employing a TOC-automatic analyser (TOC-VCSH Shimadzu).

5.2.4. DNA extraction, PCR amplification and sequencing

Microbial (bacteria and fungi) DNA was extracted from 0.25 g soil using the PowerSoil DNA Isolation Kit (MOBIO), according to the manufacturer's instructions. The isolated DNA was quantified using a NanoDrop 2000 spectrophotometer. Metagenomic analysis was performed at the Genomic Unit from the Centre for Genomic Regulation (Genomic Unit-CGR Core Facilities, Barcelona, Spain).

Two separate PCR reactions per sample were performed in order to detect bacterial 16S rDNA amplification (V3-V4 region) and fungal internal transcribed spacer 2 (ITS2) amplification. Each PCR amplification were carried out on 4 μ l DNA, 0.2 μ M of each forward and reverse primer and Kapa ready mix (KapaBio systems) in a total volume of 10 μ l. The 16S rRNA V3V4 region was amplified as previously described (Willis et al., 2018) using a set of modified primers V3-V4-F and V3-V4-R that contains a 1-4 bp "heterogeneity spacer", which was designed by the Centre for Genomic Regulation (Genomic Unit), to mitigate the issues caused by low sequence diversity amplicons. PCR conditions were modified: an initial denaturation at 95°C for 3 min, followed by 25 three-step cycles consisting of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s; and a final 5 min extension at 72°C.

The ITS2 region from DNA sample extracts were amplified using forward primer ITS3-KYO-F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGATGAAGAACGCAGCGAA-3') and reverse primer ITS4-R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCTCCGCTTATTGATATGC-3'). Samples were incubated in a thermocycler using a program of denaturing at 95°C for 3 min, followed by 35 three-step cycles consisting of 98°C for 20 s, 50°C for 30 s and 72°C for 30 s; and a final 5 min extension at 72°C.

After first PCR step, water was added to a total volume of 50 µl and reactions were purified using AMPure XP beads (Beckman Coulter) with a 0.9X (V3-V4 amplicon) or 1.2X (ITS amplicon) ratio according to manufacturer's instructions. PCR products were eluted from the magnetic beads with 32 µl of Buffer EB (Qiagen) and 30 µl of the eluate were transferred to a fresh 96-well plate. The above-described primers contain overhangs allowing the addition of full-length Nextera adapters with barcodes for multiplex sequencing in a second PCR step, resulting in sequencing ready libraries. To do so, 5 µl of the first amplification were used as template for the second PCR with Nextera XT v2 adaptor primers in a final volume of 50 µl using the same PCR mix and thermal profile as for the first PCR but only 8 cycles. After the second PCR, 25 µl of the final product was used for purification and normalization with SequelPrep normalization kit (Invitrogen), according to manufacturer's protocol. Libraries were eluted in 20 µl volume and pooled for sequencing. Final pools were quantified by qPCR using Kapa library quantification kit for Illumina Platforms (KapaBiosystems) on an ABI 7900HT real-time cycler (Applied Biosystems). Sequencing was performed in IlluminaMiSeq with 2 × 300 bp reads using v3 chemistry with a loading concentration of 18 pM.

Raw DNA sequences, both Bacteria and Fungi, were processed, by the Bioinformatics Unit from Centre for Genomic Regulation (Genomic Unit-CGR Core Facilities, Barcelona, Spain). For it was used the Mothur software (version 1.39.5) (Schloss et al., 2009), according to the standard operating protocol as described by Kozich et al., (2013). Overlapping pairs of

sequence reads were merged into contigs. In addition, reads with ambiguous bases, duplicated contigs and homopolymers longer than 13 bp were removed. The VSEARCH algorithm (embedded in the Mothur framework) was used to remove chimeras and these were subsequently omitted. The resulting sequences were classified according to the taxonomy into the corresponding Operational Taxonomic Units (OTUs) at 97 % similarity, besides using the reference trainset 16_022016.pds from https://mothur.org/wiki/RDP_reference_files for Bacteria and ITS sequences provided by the UNITE ITS database (version 7.2) at <https://unite.ut.ee/repository.php> for Fungi. Undesired lineages such as Bacteria, Plantae, Animalia, Protista, “unknown” and other were removed. The final sequences were then grouped again into OTUs, using the cluster command in Mothur, which was based on sequence similarity distances at the 0.03 and the 0.05 cut off level for Bacterial and Fungi, respectively. Finally, the resulting OTUs mapping to the same genus were grouped together.

For each study site, taxa relative abundances were calculated as the average percentage, out of three replicates, from the total count of reads in each sample. Orders (both bacteria and fungi) that showed >5 % abundance in at least one sampling site, were considered.

5.2.5. Data analysis

Statistical analyses were performed with the IBM SPSS Statistics 24 software. Homogeneity of variances was tested by using the Levene's test. Data non-normally distributed were log-transformed to fit to a normal distribution prior to statistical analyses. T-test was employed when comparing two groups, while one-way ANOVA with Tukey's test was used for comparisons among three groups. Data on bacterial and fungal orders abundance in rhizospheric samples were analysed by Canonical Correspondence Analysis (CCA) using the CANOCO software for Windows v4.02 (Ter Braak and Smilauer, 1999).

5.3. Results and discussion

5.3.1. Effects of soil properties on plant species distribution

Table 5.1 shows the edaphic characterization of the soil samples. Due to the absence of significant differences between the rhizospheric samples of the two tree species (both, at the tailings and at the control site), these samples were grouped together. Previous research showed that the distribution of spontaneous vegetation in metalliferous mine tailings is more related to edaphic properties such as pH, texture, organic matter or salinity than to metal concentrations (Párraga-Aguado et al., 2013). In fact, for some specific metals such as Zn, total concentrations obtained at the trees' rhizospheres were higher (about 1.5-fold higher, $p < 0.05$) than at the bare tailings samples, which may suggest the low relevance of this parameter regarding plant establishment. In a similar way, the 0.01 M CaCl_2 extractable metal concentrations may indicate a low effect of metal acute phytotoxicity in plant performance. For instance, previous studies have shown ranges of tolerance up to 5-6 mg L^{-1} in soil solution for metals such as Zn for *P. halepensis* (Fuentes et al., 2007), *T. articulata* (Disante et al., 2010) and *Z. fabago* (Lefèvre et al., 2010).

Table 5.1: Results of soil analyses. “1:5” refers to the results of the 1:5 soil:water extract. Data presented as the average \pm standard error of three replicates for *Z. fabago* and six for the group of *P. halepensis* and *T. articulata*. 0.01 M CaCl₂ extractable Cd and Pb concentrations were below the detection limit (<10 $\mu\text{g kg}^{-1}$). Different letters among columns of the mine tailings pile samples indicate significant differences (ANOVA with Tukey’s test, $p < 0.05$); “**” between columns of the control site indicate significant differences (T-test, $p < 0.05$); “***” between these two columns indicate significant differences (T-test, $p < 0.05$).

Parameters	Units	Mine Tailings pile			Control		
		<i>P. halepensis</i> and <i>T. articulata</i>	<i>Z. fabago</i>	Bare tailing	<i>P. halepensis</i> and <i>T. articulata</i>	<i>Z. fabago</i>	
pH (1:5)		7.4 \pm 0.1	7.2 \pm 0.1	7.2 \pm 0.0	8.0 \pm 0.0	7.8 \pm 0.0	
Electrical Conductivity (EC) (1:5)	dS m ⁻¹	1.7 \pm 0.4 a	3.5 \pm 0.1 b	3.1 \pm 0.0 b	0.2 \pm 0.0 *	1.5 \pm 0.3	
Organic Carbon (OC)	g kg ⁻¹	5.4 \pm 0.7	5.7 \pm 0.3	4.8 \pm 0.5	24.0 \pm 2.3	36.3 \pm 7.6	
Dissolved Organic Carbon (DOC) (1:5)	mg kg ⁻¹	43.7 \pm 5.4 b	18.1 \pm 2.8 a	15.9 \pm 0.9 a	166.6 \pm 13.9	417.3 \pm 65.4	
Total Nitrogen (TN)	g kg ⁻¹	0.27 \pm 0.05 b	0.24 \pm 0.07 b	0.08 \pm 0.01 a	2.42 \pm 0.18	4.97 \pm 0.86	
Particle size distribution	%	1.7 \pm 0.7 a	6.6 \pm 0.0 b	1.0 \pm 0.0 a	27.7 \pm 0.9 *	20.2 \pm 1.8	
Clay		13.0 \pm 1.8 b	42.4 \pm 0.3 c	21.3 \pm 2.7 a	32.3 \pm 1.2	35.0 \pm 3.8	
Silt		85.4 \pm 1.9 c	51.0 \pm 0.3 a	77.7 \pm 2.7 b	40.0 \pm 0.7	44.8 \pm 2.3	
Sand		9.4 \pm 0.4 a	60.1 \pm 16.7 b	14.5 \pm 1.6 a	12.3 \pm 1.4 *	110.1 \pm 15.0	
Ions in 1:5 water extract	mg L ⁻¹	958 \pm 253 a	1947 \pm 24 b	1786 \pm 35 ab	19 \pm 1 *	138 \pm 24	
SO ₄ ²⁻		34.3 \pm 2.7 b	29.3 \pm 4.4 b	8.4 \pm 0.5 a	353.6 \pm 48.0	403.4 \pm 8.4	
Microbial Biomass Carbon (MBC)	mg kg ⁻¹	0.65 \pm 0.07 b	0.50 \pm 0.04 b	0.17 \pm 0.02 a	5.12 \pm 0.26	5.69 \pm 0.09	
Dehydrogenase	$\mu\text{g INTF g}^{-1} \text{h}^{-1}$	0.27 \pm 0.06	0.14 \pm 0.03	<0.10	2.37 \pm 0.15	3.70 \pm 0.69	
β -glucosidase	$\mu\text{mol p-NP g}^{-1} \text{h}^{-1}$	34 \pm 2	23 \pm 9	33 \pm 1	<10	<10	
Total metal concentrations	mg kg ⁻¹	100 \pm 2	90 \pm 5	94 \pm 7	48 \pm 2	37 \pm 5	
Cd		10100 \pm 541	10685 \pm 1038	11523 \pm 544	1992 \pm 190 *	560 \pm 52	
Mn		4423 \pm 363 b	2620 \pm 290 a	3383 \pm 272 ab	717 \pm 160	697 \pm 36	
Pb		8359 \pm 711 b	3517 \pm 192 a	5019 \pm 382 a	418 \pm 74	352 \pm 16	
Zn		<10	55 \pm 3 **	11 \pm 1	50 \pm 4 *	96 \pm 10	
0.01 M CaCl ₂ extractable metal concentrations	$\mu\text{g kg}^{-1}$	122 \pm 17 b	116 \pm 16 b	411 \pm 111 a	111 \pm 26 *	820 \pm 140	
Cu		845 \pm 70 c	104 \pm 15 a	192 \pm 25 b	100 \pm 7 *	218 \pm 25	
Mn							
Zn							

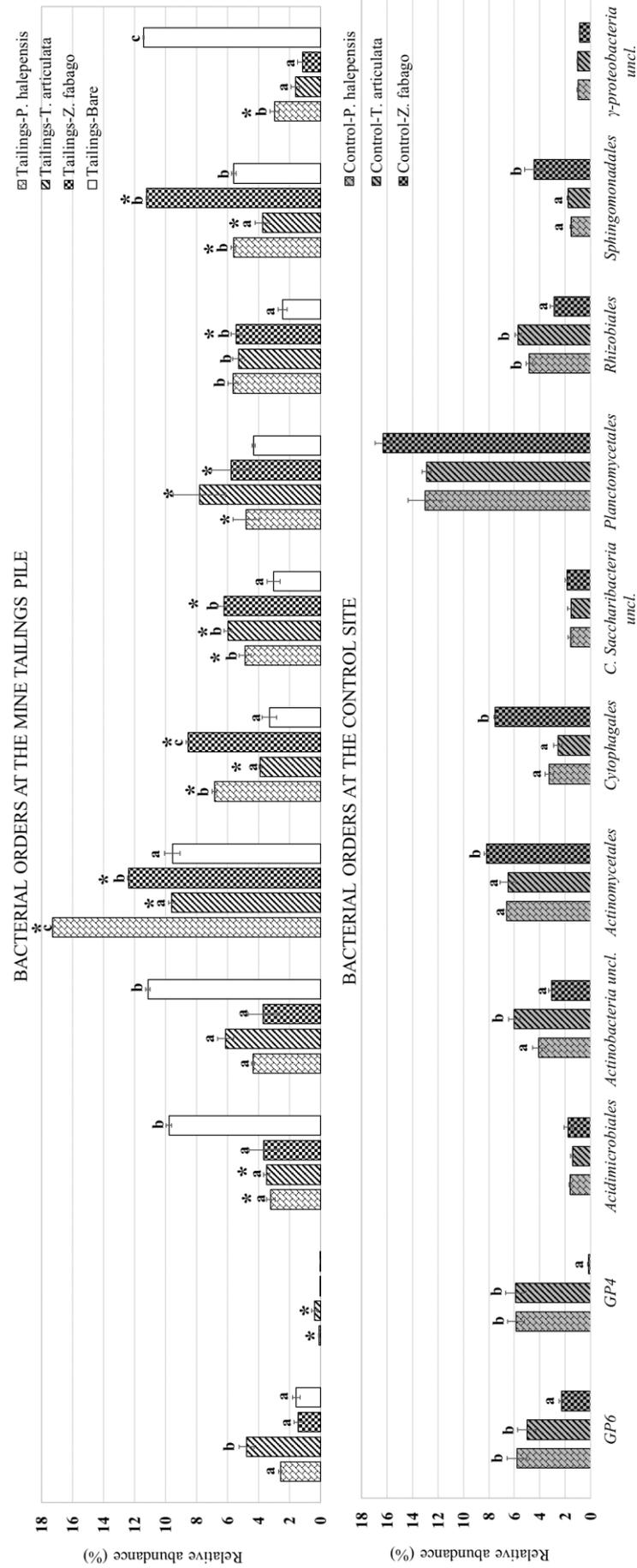
Under the neutral pH of the mine tailings substrate of our samples, due to the absence of sulphide reactive minerals (Conesa et al., 2008), other parameters such as electrical conductivity (including water extractable chloride and sulphate) or texture (particle size distribution) seemed to play an important role in plant species establishment. The specific soil conditions of patches at the mine tailings might determine the type of vegetation that spontaneously colonizes their surface (Párraga-Aguado et al., 2014b). For instance, sandier patches ($p < 0.05$) with lower salinity ($p < 0.05$, about half than that in bare tailings) resulted more favourable for trees establishment than those areas with higher electrical conductivity values and silt and clay contents, more related to the presence of *Z. fabago* ($p < 0.05$) (e.g. 2-fold higher clay and silt percentages than those in bare tailings samples). The rhizosphere of *Z. fabago* at the mine tailings showed similar EC values to those of bare tailings samples ($p < 0.05$) but contained higher chloride concentrations than the latter. As it occurred at the mine tailings, *Z. fabago* grew at the highest salinity patches at the control site, revealing the strategy of this species to get established in extreme soil conditions where less number of competitors are present (Párraga-Aguado et al., 2016). This might suggest that there was not edaphic successional link at the mine tailings pile between *Z. fabago* and the studied tree species but a simultaneous colonization following appropriate soil patches for each species. Compared to the bare tailings samples, the rhizospheric samples at the mine tailings (all species) may have stimulated the presence of nutrients (especially TN for all the species and DOC only for trees, $p < 0.05$). Moreover, the rhizospheric tailings samples showed higher MBC concentrations and higher dehydrogenase and β -glucosidase activities ($p < 0.05$ for all the species) than the bare tailings samples.

5.3.2. Plant rhizosphere effects on microbial composition

Actinobacteria (~40 %) and *Proteobacteria* (~30 %) were the most abundant phyla at the bare tailings samples. Within *Actinobacteria*, the *Actinobacteria uncl.* and *Acidimicrobiales* orders, of oligotrophic behaviour (Sun et al., 2019), showed about 3-fold higher relative abundances in the bare tailings than in the rhizosphere tailings samples, both for trees and *Z.*

fabago (Figure 5.3, $p < 0.05$). Within the *Proteobacteria* phylum, the main contributor at the bare tailings samples was the γ -*proteobacteria uncl.* order (abundance ~13 %). That higher abundance of the γ -*proteobacteria* taxa in the bare tailings than in the rhizosphere samples (about 4-fold higher, $p < 0.05$, Figure 5.3) may be explained by its antagonistic interaction or competition with plants for available nutrients (Sun et al., 2018). However, the other groups of *Proteobacteria*, which belonged to the α -*proteobacteria* class, showed similar or higher abundance at the rhizospheres than at the bare tailings samples. Similarly to the results reported by Sun et al. (2018), we found some orders of γ -*proteobacteria* more related to bare tailings samples and some α -*proteobacteria* orders, such as *Rhizobiales* and *Sphingomonadales*, more abundant in vegetated tailings samples (Figure 5.3). In this sense, several studies have concluded that rhizospheric α -*proteobacteria* taxa of spontaneous vegetation at mine tailings play an important role in the stimulation of biogeochemical cycle of N (Herrera et al., 2007; Wakelin et al., 2012). In addition, several studies have revealed the importance of the *Rhizobiales* order in the enhancement of plants capacity to cope with abiotic stress factors such as high soil metal(loid) concentrations (Chen et al., 2008; Hao et al., 2014) or extreme saline conditions (Etesami and Beattie, 2018).

Figure 5.3: Bacterial relative abundance for each sampling site. Results are presented as the average percentage of three replicates. Bars on columns indicate standard errors. Different letters indicate statistical significance among sampling sites (ANOVA with Tukey's test, $p < 0.05$). Asterisks on columns indicate, for each plant species, significant differences between samples of the mine tailings and the control site (T-test, $p < 0.05$).



Compared to the control site, rhizospheres at the mine tailings showed significantly lower abundance values of *Planctomycetales* (Figure 5.3 and Figure 5.4). This bacterial order have been shown to be sensitive to heavy metals (Fuerst and Sagulenko, 2011; Oka and Uchida, 2018). On the other hand, rhizospheres at the mine tailings showed significantly higher abundance percentages of *Actinomycetales*, *Sphingomonadales*, *Cytophagales* and *Candidatus Saccharibacteria uncl.* (Figure 5.3 and Figure 5.4). For the case of the *Cytophagales* and *Actinomycetales* orders, this fact could be explained by their ability to colonize plant roots in poor nutrient soils (Zarraonaindia et al. (2015) and Colin et al. (2017), respectively). In addition, taxa belonging to the *Actinomycetales* order may facilitate the adaptation of plants to saline soils (Battini et al., 2017; Etesami and Beattie, 2018) even for those species that do not show a remarkable halotolerant behaviour (Siddikee et al., 2010, 2011). This may explain the higher abundance of the *Actinomycetales* order in the rhizospheric mine tailings samples compared to those of the control site for the three plant species (Figure 5.3).

The results of the CCA for the bacterial orders are shown in the Figure 5.4 and Tables 5.2 and 5.3. The positive side of the CCA1-axis was defined by higher values of some soil fertility parameters ($r > 0.50$, OC, TN, MBC, dehydrogenase, β -glucosidase). This may have favoured higher abundances of the nutrient recycler *Planctomycetales* taxa (Nie et al., 2015; Oka and Uchida, 2018), which are known to proliferate in soils with available supply of organic carbon (Meglouli et al., 2018). The negative side of the CCA1-axis was conditioned by higher values of total metal concentrations ($r < -0.6$) and salinity ($r \sim -0.7$, for EC and SO_4^{2-}), in addition to similarly elevated abundances of oligotrophic bacteria such as *Acidimicrobiales* (Sun et al., 2019) or plant growth promoting bacteria such as *Candidatus Saccharibacteria uncl.* (Navarro-Noya et al., 2010; Ni et al., 2016). The CCA1-axis segregated those bacterial orders of all the mine tailings rhizospheres from those tree rhizospheres developing in the control site. The CCA2-axis indicated the influence of fertility parameters on the positive side ($r > 0.50$ for OC, DOC, TN, CBM, β -glucosidase) together with higher concentrations of water extractable Cl^- and 0.01 M

CaCl₂ extractable Mn. Also, the CCA2-axis segregated the rhizospheric samples of *Z. fabago* at the control site from the rest of the samples.

Table 5.2: Data of the Canonical Correspondence Analysis (CCA) including eigenvalues of the two first axis and cumulative percentage of variance of bacteria-soil data interaction. The significance of the first canonical axis (Monte-Carlo test, p-value) is also provided.

Parameter	Axis	Eigenvalue	% cumulative variance	Significance
Bacterial orders	I	0.167	65.5	0.1980
	II	0.047	83.8	

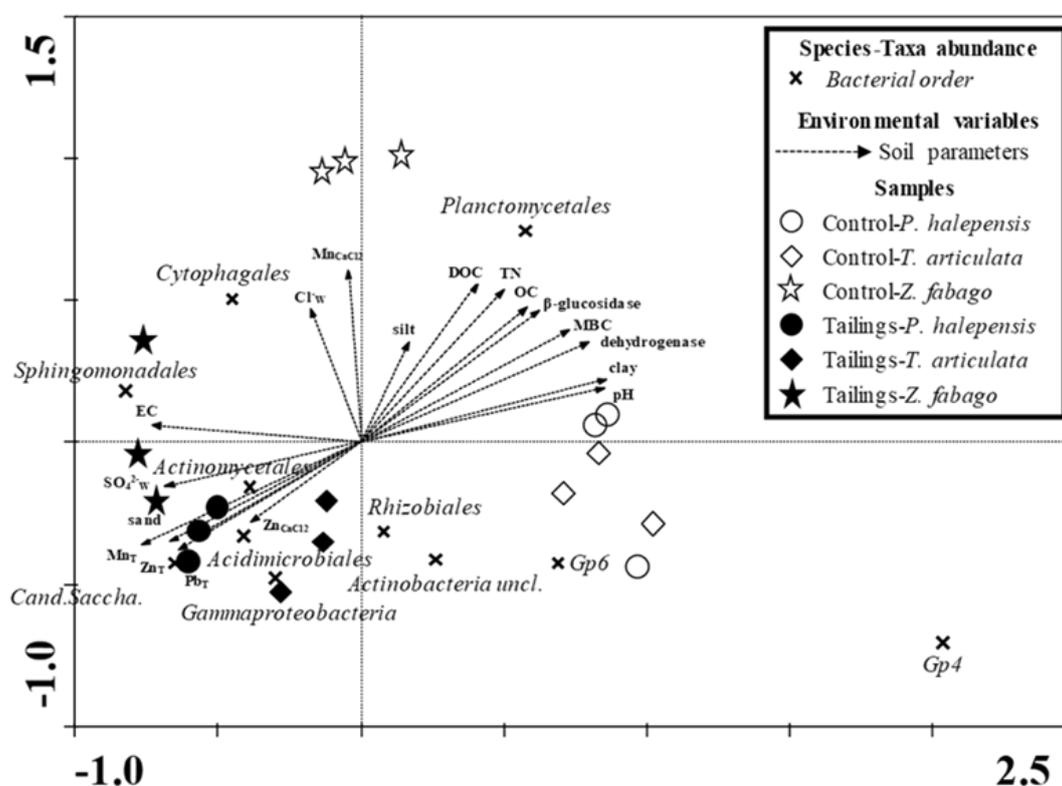


Figure 5.4: Ordination diagram obtained for the Canonical Correspondence Analysis (CCA) of bacterial orders abundance and soil properties. “Mt” are total concentrations of the M element; “SO₄^{2-w}” and “Cl_w” are water extractable sulphate and chloride concentrations, respectively; “M_{CaCl2}” are 0.01 M CaCl₂ extractable concentrations of the M element; “OC” is Organic Carbon; “TN” is Total Nitrogen concentrations; “DOC” is Dissolved Organic Carbon; “Sand”, “Silt” and “Clay” are the corresponding percentages of particle size; “EC” and “pH” are Electrical Conductivity and pH, respectively, of the 1:5 soil:water extract; “MBC” is the Microbial Biomass Carbon; dehydrogenase and β-glucosidase are enzymatic activities.

Plant biogeochemical processes and their exudates may condition the microbial composition of the rhizosphere (Colin et al., 2017). On the other hand, trees might result more dependent on long-term processes such as nutrient cycling, litter decomposition, etc, compared to herbaceous annual plants, which may also determine different functionalities of the rhizospheric microbiome. At the control site, bacterial composition for both tree species were similar (only differed in *Actinobacteria uncl.*, Figure 5.3) and were mainly dominated by *Planctomycetales* (12-16 %, Figures 5.3 and 5.4), while at the mine tailings, significant differences occurred in five orders (Figure 5.3). This may be explained because at the control site the trees formed part of a dense forest, where the influence of individual rhizospheres may be modulated by the neighbouring effect of other co-occurring plant species. However, at the mine tailings, the trees grew isolated and it is likely that their rhizospheres could act as a “filter” for a more specific bacterial composition. In this regard, the rhizosphere of *T. articulata* at the mine tailings showed lower abundances of nutrient recycling and plant growth promoting bacteria such as *Actinomycetales*, *Sphingomonadales* or *Cytophalages* than the other two plant species. These differences may be related to the ability of *T. articulata* roots to release specific allelochemicals, with inhibitory growing effects for plant competitors (M’barek et al., 2018; Rached et al., 2018), that may have affected negatively those plant growth promoting bacteria.

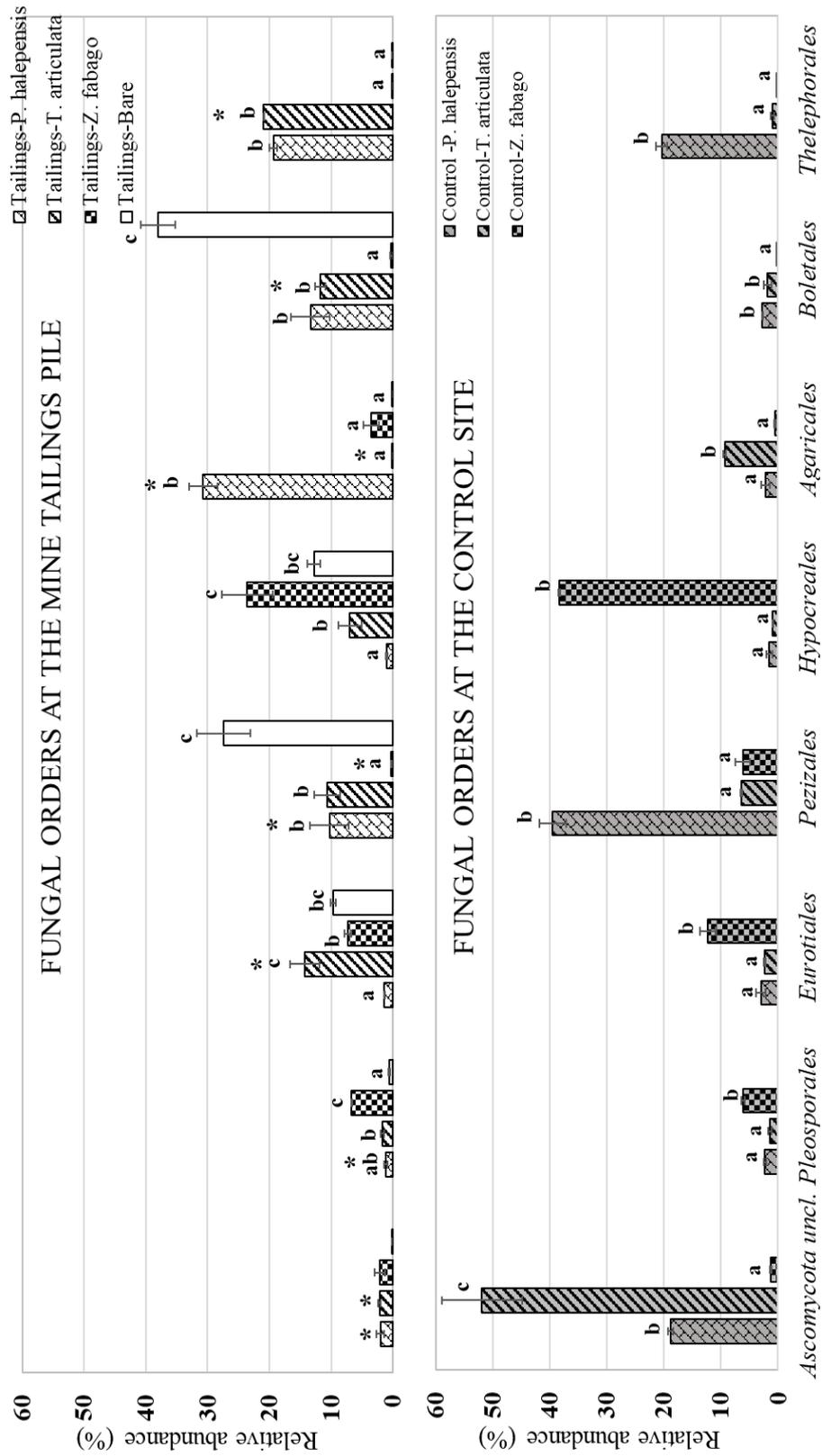
Table 5.3: Weighted correlation matrix for the first two species axes and environmental variables for bacterial orders.

Soil parameter		Bacterial orders	
		Species Ax1	Species Ax2
pH		0.86	0.27
Electrical Conductivity (EC)		-0.74	0.08
Organic Carbon (OC)		0.59	0.66
Dissolved Organic Carbon (DOC)		0.41	0.78
Total Nitrogen (TN)		0.51	0.75
Particle size distribution	Clay	0.87	0.31
	Silt	0.17	0.49
	Sand	-0.59	-0.47
0.01 M CaCl ₂ metal extractable concentrations	Mn	-0.04	0.84
	Zn	-0.39	-0.40
Total metal concentrations	Mn	-0.78	-0.51
	Pb	-0.68	-0.49
	Zn	-0.65	-0.53
Water extractable ions	Cl ⁻	-0.18	0.66
	SO ₄ ²⁻	-0.70	-0.22
Microbial Biomass Carbon (MBC)		0.74	0.55
Enzymatic activities	dehydrogenase	0.81	0.49
	β-glucosidase	0.63	0.65

Halophytic plant species are able to shape specific microbial composition in their rhizospheres that may enhance plant growth (Etesami and Beattie, 2018). This could explain the higher abundance detected of *Sphingomonadales* and *Cytophagales* in the *Z. fabago* rhizosphere, both at the control and at the tailings, compared to the rest of samples (including the bare tailings). For instance, taxa belonging to the *Sphingomonadales* order are known to develop within the rhizospheres of halophytes in salt marshes (Oliveira et al., 2014) and to enhance the plant ability to mobilize K from soil, favouring its uptake (Etesami and Alikhani, 2019). On the other hand, several taxa of the *Cytophagales* order have been shown to associate with halophytes, such as *Arthrocnemum macrostachyum*, a typical plant species in Mediterranean salt marshes (Camacho-Sanchez et al., 2020).

In relation to fungal composition, the bare tailings samples were mainly composed by the *Boletales* (~38 %, *Basidiomycota* phylum) and *Pezizales* (~28 %, *Ascomycota* phylum) orders (Figure 5.5). The rhizospheric samples of both trees species at the mine tailings showed lower abundances of *Ascomycota uncl.* ($p < 0.05$) than the control site. For the rest of the identified fungal orders, each tree species exhibited different fungal composition at the mine tailings rhizospheres compared to their corresponding control site rhizospheres. For instance, the rhizospheric tailings samples of *T. articulata* showed significantly higher values ($p < 0.05$) of the relative abundances of *Thelephorales*, *Boletales* and *Eurotiales* orders and significantly lower ($p < 0.05$) of the *Pezizales* and *Agaricales* orders than the control samples. In the case of the *P. halepensis*, the rhizospheric tailings samples showed lower abundance percentages ($p < 0.05$) of the *Pleosporales* and *Pezizales* orders and higher abundance values ($p < 0.05$) of the *Agaricales* than the control rhizospheres. *Zygothellium fabago* only differed in the *Pezizales* order, whose relative abundance values were lower ($p < 0.05$) at the mine tailings rhizospheres than at the control samples.

Figure 5.5: Fungal relative abundance for each sampling site. Results are presented as the average percentage of three replicates. Bars on columns indicate standard errors. Different letters among columns indicate significant differences among samples at each sampling site (ANOVA with Tukey's test, $p < 0.05$). Asterisks on columns indicate for each plant species the occurrence of significant differences between samples of the mine tailings and the control site (T-test, $p < 0.05$).



The results of the CCA for the fungal orders are shown in the Figure 5.6 and Tables 5.4 and 5.5. The CCA1-axis was defined in the positive side by salinity ($r \sim 0.7$ for EC, $r \sim 0.5$ for water extractable Cl^- and SO_4^{2-}), segregating the rhizospheric samples of *Z. fabago* from the tree samples. The fungal community within the rhizospheres of *Z. fabago* was mainly composed of three fungal orders: *Eurotiales* at the control site and *Pleosporales* and *Hypocreales*, both at the control site and at the mine tailings. These three fungal orders also resulted the most abundant in the rhizosphere of *Hordeum murinum*, another ruderal plant species, when growing at urban and suburban saline soils (Murphy et al., 2015). The similar fungal composition between these two ruderal plant species may be explained by the ability of the mentioned fungal orders to promote plant growth in saline environments (Murphy et al., 2015) and their behaviour as endophytes for most of plants (Kolaříková et al., 2017). However, the low abundances found for those three fungal orders at both tree species may be attributed to their low affinity to grow within the rhizospheres of mycorrhizal plant dependent species (Glynou et al., 2016), such as the case of the trees *P. halepensis* and *T. articulata*.

Table 5.4: Data of the Canonical Correspondence Analysis (CCA) including eigenvalues of the two first axis and cumulative percentage of variance of fungal-soil data interaction. The significance of the first canonical axis (Monte-Carlo test, p-value) is also provided.

Parameter	Axis	Eigenvalue	% cumulative variance	Significance
Fungal orders	I	0.584	45.6	0.004
	II	0.376	74.9	

Table 5.5: Weighted correlation matrix for the first two species axes and environmental variables for fungal orders.

Soil parameter	Fungal orders		
	Species Ax1	Species Ax2	
pH	-0.31	0.63	
Electrical Conductivity (EC)	0.71	-0.27	
Organic Carbon (OC)	0.17	0.43	
Dissolved Organic Carbon (DOC)	0.38	0.37	
Total Nitrogen (TN)	0.30	0.44	
Particle size distribution	Clay	-0.20	0.72
	Silt	0.41	0.76
	Sand	-0.11	-0.83
0.01 M CaCl ₂ metal extractable concentrations	Mn	0.65	0.15
	Zn	-0.10	-0.75
Total metal concentrations	Mn	0.03	-0.61
	Pb	-0.07	-0.68
	Zn	-0.14	-0.77
Water extractable ions	Cl ⁻	0.48	-0.14
	SO ₄ ²⁻	0.44	-0.32
Microbial Biomass Carbon (MBC)		-0.03	0.67
Enzymatic activities	dehydrogenase	-0.04	0.69
	β-glucosidase	0.15	0.58

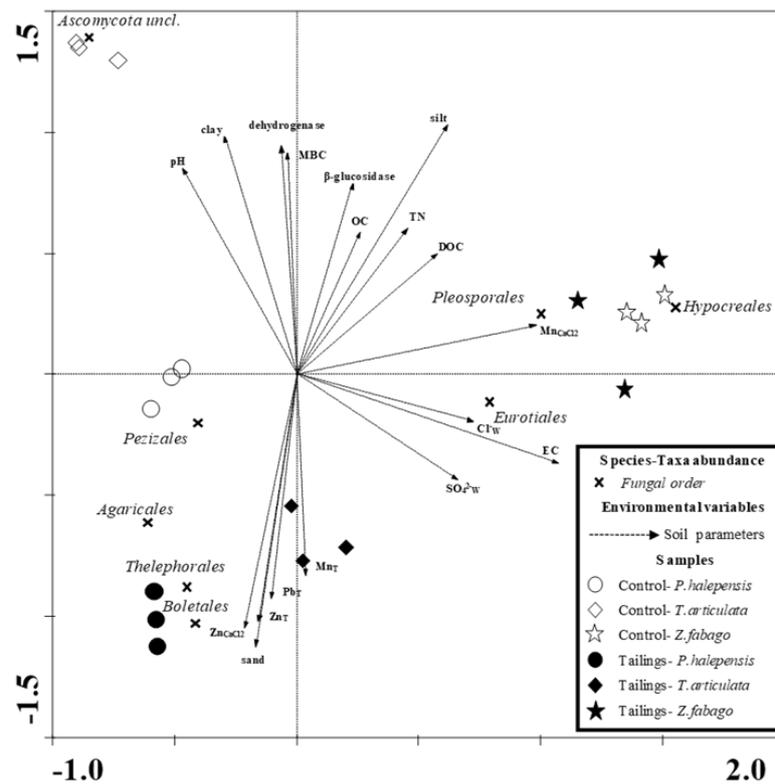


Figure 5.6: Ordination diagram obtained for the Canonical Correspondence Analysis (CCA) of fungal orders abundance and soil properties. “Mt” are total concentrations of the M element; “ SO_4^{2-w} ” and “ Cl_w ” are water extractable sulphate and chloride concentrations, respectively; “ M_{CaCl_2} ” are 0.01 M CaCl_2 extractable concentrations of the M element; “OC” is Organic Carbon; “TN” is Total Nitrogen concentrations; “DOC” is Dissolved Organic Carbon”; “Sand” “Silt” and “Clay” are the corresponding percentages of particle size; “EC” and “pH” are Electrical Conductivity and pH, respectively, of the 1:5 soil:water extract; “MBC” is the Microbial Biomass Carbon; dehydrogenase and β -glucosidase are enzymatic activities.

The rhizospheric samples from tailings and the control site belonging to *P. halepensis* and *Z. fabago* did not segregate (Figure 5.6, samples of each species appeared at the same quadrant). This may indicate the strong effect of their rhizospheres on determining fungal composition. *Tetraclinis articulata* showed a high segregation between samples of the tailings and the control area, indicating a weaker effect of its rhizosphere on fungal composition (samples of *T. articulata* at different quadrants) than for the other two species. In addition, the contrasting mycorrhizal behaviour of both tree species could explain the differences found between their fungal rhizospheric composition: while *P. halepensis* is known to be associated to ectomycorrhizal fungi (Querejeta et al., 1998), *T. articulata* is an endomycorrhizal dependant

species (Abbas et al., 2006). In this way, the abundances of typical ectomycorrhizal orders such as *Pezizales*, *Agaricales* or *Thelephorales* (Tedersoo et al., 2010) were higher in the rhizospheres of *P. halepensis* compared to those found for *T. articulata*.

Although most taxa belonging to the *Ascomycota* order behave as ectomycorrhizal fungi, it may also contain some specific endomycorrhizal taxa (Brooks-Gould, 2008). This could explain the remarkable high abundance of *Ascomycota uncl.* within the rhizosphere of *T. articulata* (an obligated endomycorrhizal plant species) at the control site. The similar abundances of representative ectomycorrhizal orders, such as *Thelephorales* or *Boletales*, found at the mine tailings rhizospheres of *T. articulata* and *P. halepensis* could be attributed to the presence of those taxa in the mine tailings substrate and not necessarily as an associated symbiosis with the endomycorrhizal species *T. articulata* (Toju and Sato, 2018). Finally, the higher abundance of the *Boletales* order in the bare tailings samples compared to the rhizospheres could be explained by its remarkable competitive behaviour to proliferate in those bare polluted substrates (with less number of competitors) than within plant rhizospheres (Mikryukov et al., 2020).

5.4. Conclusions

The comparison among the edaphic properties, including microbiology, associated to the rhizospheres of the spontaneous vegetation at metal enriched tailings revealed different strategies of colonization. Under the neutral pH of the tailings, metal concentrations played a secondary role in determining plant establishment. While the ruderal *Z. fabago* selectively colonized the highest salinity patches, both at the tailings and at the control site, the trees species, *P. halepensis* and *T. articulata*, grew at moderate salinity patches of the tailings. This may suggest that the establishment of early ruderal colonizers such as *Z. fabago* could be limited to areas with most adverse soil conditions of the mine tailings, while late successional plant species may selectively colonize more favourable tailings patches. In terms of phytomanagement, this selective plant species colonization of tailings would allow increasing their biodiversity and thus their resilience against environmental stressors.

Specific halotolerant bacterial and fungal taxa at the tailings plant rhizospheres might result a key factor to facilitate the establishment of vegetation at neutral pH tailings. Further works may focus on the implementation of practices to promote, not only better nutrient edaphic conditions through the addition of soil amendments, but also, a specific microbiome in order to cope the abiotic stress of plants at mine tailings.

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**CHAPTER 6. A critical assessment on the short-term
response of microbial relative composition in a mine tailings
soil amended with biochar and manure compost**

A critical assessment on the short-term response of microbial relative composition in a mine tailings soil amended with biochar and manure compost

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Abstract

Phytomanagement of tailings requires the use of soil conditioners to favour plant establishment, but their benefits on soil microbial composition need to be assessed. The goal of this work was to evaluate the effect of two organic amendments, manure compost and biochar, on soil bacterial and fungal composition at metallic mine tailings. The addition of compost caused stronger effects in most of soil parameters and microbial composition than biochar, especially at the initial stage of the experiment. However, the higher dependence on labile organic carbon for some bacterial groups at the treatments containing compost determined their decay along time (*Flavobacteriales* and *Sphingobacteriales*) and the appearance of other taxa more dependent on recalcitrant organic matter (*Xanthomonadales* and *Myxococcales*). Biochar favoured bacterial decomposers (*Actinomycetales*) specialized in high lignin substrates and other recalcitrant carbon compounds. Unlike bacteria, only a few fungal orders increased their relative abundances in the treatments containing compost (*Sordariales* and *Microascales*), while the rest showed a decrease or remained unaltered. The mix biochar-compost may result the best option to support a more diverse microbial population in terms of soil functionality that is able to decompose both labile and recalcitrant carbon compounds. This may favour the resilience of the system against environmental stressors.

6.1. Introduction

Mine tailings are wastes composed by the left-over of ore-processing activities and they are considered as the main responsible for environmental health impacts in former metallic mining areas (Conesa and Schulin, 2010). The bare areas of these tailings are usually impacted by wind and water erosion, which may spread metal(loid) enriched particles to their surroundings. Phytomanagement by phytostabilization is considered a suitable alternative to decrease the environmental risks associated to mine tailings (Robinson et al., 2009). This technique consists of the generation of a stable vegetation cover at the tailings surfaces, which decreases the erosion through the fixation of soil particles within plant rhizospheres (Wong, 2003; Mendez and Maier, 2008).

Soil conditions within tailings are unfavourable for plant growth (e.g. high metal(loid) concentrations, low fertility, high salinity). To overcome this issue, the addition of soil conditioners that ameliorate soil constraints are usually proposed (Clemente et al., 2010; Párraga-Aguado et al., 2015). Among the available options, those including organic amendments are highly appreciated because of their beneficial effects in soil fertility and metal(loid) mobility reduction (Clemente et al., 2010; Pardo et al., 2014a, 2014b). The final performance of these amendments depends on their nature, the rate of addition and the edaphic properties of the polluted soil. The combined use of organic amendments like compost and biochar has been suggested as an interesting option in mine tailings because of their complementarity: composts are known to provide readily available nutrients for plants; biochar is a type of organic material obtained by pyrolysis that shows high capacity to complex metals (because of its high specific surface area and negative sorption sites) and high stability to be degraded (due to its high content of recalcitrant organic carbon) (Rodríguez-Vila et al., 2017; Forján et al., 2018). Although there is a general acceptance of the benefits associated to the addition of organic amendments in tailings, recent works consider necessary to carry out a specific assessment to avoid any undesirable effects such as increases of metal(loid) available pools or negative impacts in plant ecological relationships (Pardo et al., 2014a; Martínez-Oró et al., 2019).

Recent works on the phytomanagement of mine tailings have pointed out the critical role of soil microbiology for the successful establishment of vegetation (Kolaříková et al., 2017; Sun et al., 2018). Soil microorganisms may interact with plants rhizospheres, acting as filters to decrease the phytotoxic effects of metal(loid)s and as supporters of C-N cycles, providing available nutrients for plant growth (Thavamani et al., 2017). The adverse edaphic conditions at tailings may restrict the native microbial composition to those tolerant or highly resistant groups but with limited soil functional capabilities (Risueño et al., 2020a, Chapter 4). Nevertheless, and in spite of these limitations, those bacterial or fungal groups could play an important role in favouring the edaphic successional processes, which might support the establishment of a self-sustaining vegetation (Kolaříková et al., 2017; Sun et al., 2018; Colin et al., 2019). A key factor in these systems could be to accelerate these successional processes driven by soil microbiology through the application of organic amendments. The assessment of the effects of soil amendments is then critical, because the autochthonous microbiology of the tailings, already adapted to high metal(loid) concentrations, salinity and low fertility conditions, might be negatively impacted by those microorganisms contained in the amendments or by the changes in soil conditions, which the amendments might generate (Grandlic et al., 2008). In addition, it is important to assess whether the microbiome contained in the amendment is negative impacted by the edaphic conditions of the tailings or not.

The goal of this work was to assess the effect of the single application of two organic amendments, manure compost, as a source of labile carbon, and biochar, as a recalcitrant organic carbon material, and their combination, in the soil bacterial and fungal composition within a metal enriched mine tailings soil. For that purpose, a dynamic mesocosm experiment was carried out comparing four different treatments: bulk tailings, bulk tailings + compost, bulk tailings + biochar and bulk tailings + compost + biochar. Soil parameters data (pH, electrical conductivity, dissolved organic carbon, dissolved nitrogen, water extractable ions and metals) and soil microbial composition data (bacteria and fungi) were collected and analysed at three different times from the beginning of the experiment: 1 month after, 3 months after and 6 months after.

6.2. Material and methods

6.2.1. Characterization of mine tailings soil and amendments (compost and biochar)

The mine tailings substrate was taken at a former mine tailings disposal site located at the former Mining District of Cartagena-La Unión (southeast of Spain, 0-385 m a.s.l.; 37° 37' N, 0° 49' W– 37° 35' N, 0° 50' W, ~ 50 km²) (Figure 6.1). Former mining activities focused on metallic sulphur minerals such as galena, pyrite or sphalerite. The local climate is semiarid, with annual rainfall of 250-300 mm, and average temperature of 18°C. Additional information on the environmental impacts of the mining activity in this area is available in Conesa and Schulin (2010). The amendments used in this study consisted of biochar (B) and composted manure (C). The biochar, acquired from *Proiniso S.A.* (Málaga, Spain), was produced from tree wood (a mix of pine, oak and eucalyptus) after pyrolyzation at 900°C. The composted manure, provided by local farmers, consisted of a mixture of chicken, horse and sheep dung that was composted during three months in open air piles.

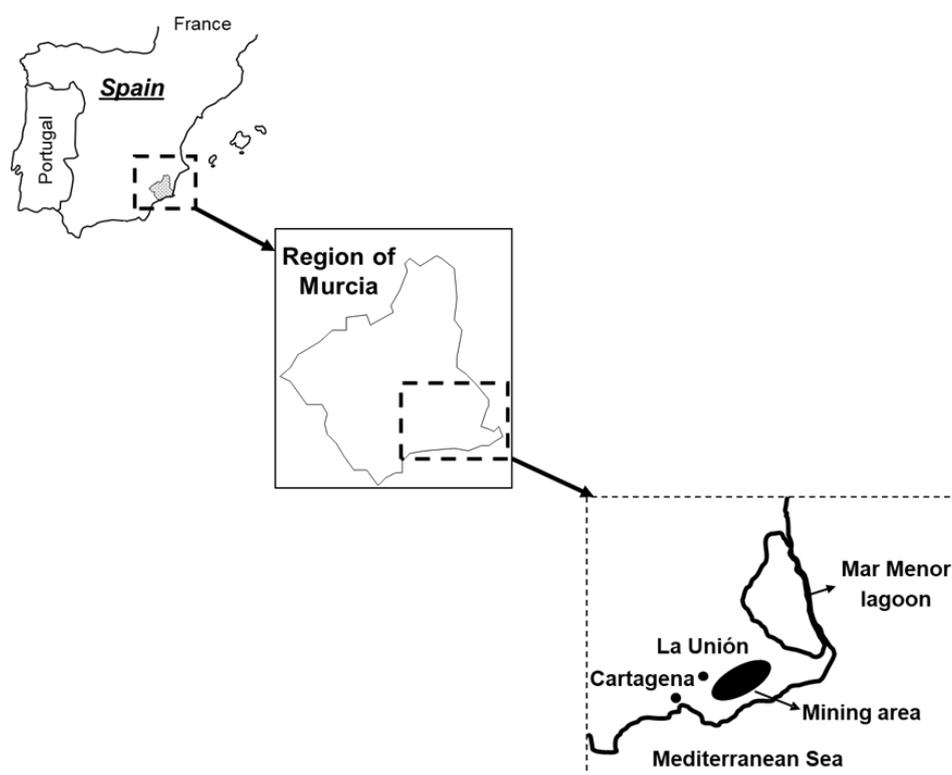


Figure 6.1: Location of the studied area.

Mine tailings soil and amendments were sieved through a 2 mm mesh. Some of the properties of the tailings substrate and the two amendments are shown in Table 6.1.

Table 6.1: Results of initial characterization of the mine tailings soil and the two amendments used in this study (compost and biochar). pH, EC, DOC, DN and ions were determined in the 1:2.5 soil:water extract. Water extractable ions concentrations were determined by using an Ion Chromatographer (Metrohm), DOC and DN by using a TOC-automatic analyser (TOC-VCSH Shimadzu) and TOC and TN were determined in solid samples by a CHN 628 Leco analyser. Total metal(loid) concentrations were determined by X-ray fluorescence (Bruker S4 Pioneer). Data are average \pm standard error of 3 pseudo-replicates.

Soil parameters	Units	Mine Tailings	Compost	Biochar
pH	-	7.38 \pm <0.1	8.17 \pm <0.1	9.90 \pm <0.1
Electrical Conductivity (EC)	dS m ⁻¹	2.07 \pm <0.1	9.75 \pm 0.11	2.65 \pm <0.1
Total Organic Carbon (TOC)	%	0.45 \pm 0.01	25.80 \pm 0.20	82.90 \pm 0.04
Disolved Organic Carbon (DOC)	mg kg ⁻¹	10.8 \pm 0.5	4490 \pm 153	790 \pm 55
Total Nitrogen (TN)	%	0.24 \pm 0.01	2.50 \pm 0.01	0.70 \pm 0.01
Dissolved Nitrogen (DN)	mg kg ⁻¹	<2.5	1244 \pm 28	8.7 \pm 0.2
Ions water extract				
	Cl ⁻ mg kg ⁻¹	14 \pm 1	4537 \pm 250	130 \pm 10
	SO ₄ ²⁻	4130 \pm 40	3711 \pm 194	110 \pm 6
	Ca ²⁺	1613 \pm 11	387 \pm 38	190 \pm 26
	K ⁺	15 \pm 2	6011 \pm 169	4720 \pm 270
	Mg ²⁺	87 \pm 5	175 \pm 21	70 \pm 17
	Na ⁺	10 \pm <1	1889 \pm 50	230 \pm 14
Total elemental concentrations				
	Ca %	7.246 \pm 0.132	22.538 \pm 0.138	7.077 \pm 0.074
	K	0.604 \pm 0.011	4.360 \pm 0.035	1.871 \pm 0.018
	Mg	1.283 \pm 0.043	1.333 \pm 0.013	0.533 \pm 0.003
	Na	0.102 \pm 0.015	0.689 \pm 0.013	0.071 \pm 0.003
	P	0.037 \pm 0.001	2.428 \pm 0.015	0.143 \pm <0.001
Total metal(loid) concentrations				
	Cu mg kg ⁻¹	163 \pm 6	331 \pm 5	67 \pm 1
	Mn	11973 \pm 202	1295 \pm 14	960 \pm 13
	Pb	10041 \pm 386	77 \pm 6	<10
	Zn	14913 \pm 409	1255 \pm 16	100 \pm 1

Tailings substrate showed neutral pH (7.38), moderate electrical conductivity (2.07 dS m⁻¹), mainly due to sulphate contribution (4130 mg kg⁻¹ water extractable SO₄²⁻), low values of organic matter (e.g. < 0.5 % total organic carbon) and high total metal concentrations (e.g. > 10 000 mg kg⁻¹ Zn, Mn and Pb). Compost showed alkaline pH (8.17), high electrical conductivity (9.75 dS m⁻¹), mainly due to the contribution of chloride concentrations (~ 4500 mg kg⁻¹ water extractable Cl⁻), high total Ca (~ 22 %), K (~ 4 %), organic carbon (~ 25 %) and nitrogen (~ 2.5 %) concentrations, and some moderate total metal concentrations (e.g. 1255 mg kg⁻¹ Zn, 1295 mg kg⁻¹ Mn). Biochar was strongly alkaline (pH 9.90), with moderate electrical conductivity (2.65 dS m⁻¹), high total Ca (~ 7 %), K (~ 1.8 %) and organic carbon (~ 83 %) concentrations, low total

nitrogen (0.70 %) and low total metal concentrations (e.g. $100 \pm 1 \text{ mg kg}^{-1} \text{ Zn}$, $< 10 \text{ mg kg}^{-1} \text{ Pb}$). When comparing both amendments, in terms of labile organic matter, the compost showed higher concentrations of dissolved organic carbon ($4490 \text{ mg kg}^{-1} \text{ DOC}$) than the biochar ($790 \text{ mg kg}^{-1} \text{ DOC}$). This difference also occurred for the dissolved nitrogen concentration ($1244 \text{ mg kg}^{-1} \text{ DN}$ in the compost; $8.7 \text{ mg kg}^{-1} \text{ DN}$ in the biochar).

6.2.2. Experimental set-up

Four treatments were carried out (Figure 6.2): S, corresponded to the bulk tailings substrate; SC consisted of bulk tailings soil and 4 % (weight) of compost; SB was composed by bulk tailings soil and 4 % (weight) of biochar; and SCB was the treatment combining bulk tailings with 4 % of biochar and 4 % of compost. Plastic pots (13 cm diameter, 15 cm height), nine for each treatment, were filled with $\sim 1.5 \text{ kg}$ of treatment substrates and randomly distributed in a climate chamber with controlled temperature/light/humidity (23°C during 16h light and 16°C during 8h darkness; 60 % constant relative humidity). Pots were watered approximately at half of field capacity throughout the experiment with distilled water.

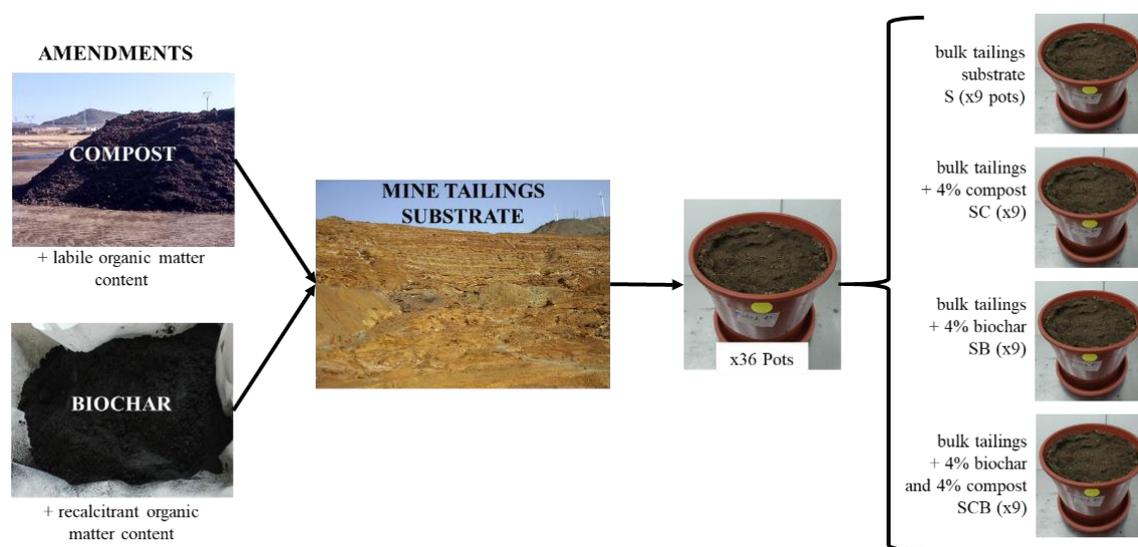


Figure 6.2: Scheme of the treatments carried out.

Soil samples were collected at three different points: after one month (T1 samples) from the beginning of the experiment, after three months (T3 samples) and after six months (T6 samples). At each sampling point, a composite soil sample each three pots ($\sim 12 \text{ g}$) was collected

in a sterilized plastic cylinder, resulting in three composite soil samples per treatment and sampling time. An aliquot of each sample was stored at -2°C for microbial analysis. The rest was used for soil parameters determination.

6.2.3. Soil parameters analyses

For all samples, a 1:2.5 soil (g):water (ml) extraction was performed by shaking for 2 hours. These extracts were filtered through nylon membrane $0.45\ \mu\text{m}$ syringe filters (WICOM) and used for measuring pH, Electrical Conductivity (EC), water extractable ions (K^+ , Mg^{2+} , Ca^{2+} , Cl^- and SO_4^{2-}) by using an Ion Chromatographer (Metrohm), Dissolved Organic Carbon (DOC) and Dissolved Nitrogen (DN) by using a TOC-automatic analyser (TOC-VCSH Shimadzu) and metals (Cu, Mn, Pb, Zn) by using an ICP-MS (Agilent 7500A).

6.2.4. DNA extraction, PCR amplification and sequencing

Microbial (bacteria and fungi) DNA was extracted from 0.25 g soil using the PowerSoil DNA Isolation Kit (MOBIO), according to the manufacturer's instructions. The isolated DNA was quantified using a NanoDrop 2000 spectrophotometer. Library preparation and Illumina sequencing were carried out at the IPBLN Genomics Facility (CSIC, Granada, Spain). Amplicon libraries targeting the V3-V4 region of 16S rDNA (bacteria) and ITS2 region (fungi) were generated by a two-steps PCR strategy. Gene-specific amplification was performed in triplicate with 15 ng of soil-extracted DNA in a final volume of $10\ \mu\text{l}$. Gene specific primers, V3V4fw (5' CCTACGGGNGGCWGCAG 3'), V3V4rev (5' GACTACHVGGGTATCTAATCC 3'), ITS3_KYO2-Fw (5' GATGAAGAACGYAGYRAA 3') and ITS4-Rev (5' TCCTCCGCTTATTGATATGC 3'), were designed with Nextera overhang adapters. Primers were used at a final concentration of $0.2\ \mu\text{M}$. Reaction was performed with 1x KAPA HiFi Hot Start Ready Mix DNA polymerase (Roche Diagnostics, West Sussex, United Kingdom). Cycling conditions were 95°C for 3 min; 25 x (95°C for 30 s, 55°C for 30 s, 72°C for 30 s) and then 72°C for 5 min for 16S amplification and 95°C for 3 min; 27 x (95°C for 30 s, 47°C for 30 s, 72°C for

30 s) and then 72°C for 5 min for ITS2 amplification. Triplicates were pooled together and validated through visualization on a 1.8 % (w/v) agarose gel. Amplicons were then purified using NucleoMag® NGS Clean-up and Size Select Kit (Macherey-Nagel, Düren, Germany). A second PCR step attached dual combinatorial indices and Illumina sequencing adapters using Nextera XT v2 index kit. Cycling conditions were 95°C for 3 min, 8 x (95°C for 30 s, 55°C for 30 s, 72°C for 30 s) and then 72°C for 5 min. Amplicon generation was validated again through visualization on a 1.8 % (w/v) agarose gel and cleaned with NucleoMag® NGS Clean-up and Size Select Kit (Macherey-Nagel). Concentration was measured on the Qubit® fluorometer (Thermo). Amplicons were pooled in an equimolecular manner and final library mix was run on a Bioanalyzer HS DNA chip to verify quality and size distribution. The library pool was then diluted and denatured as recommended by Illumina MiSeq library preparation guide. The 300x2nt paired-end sequencing was conducted on a MiSeq sequencer.

6.2.5. Bioinformatics and statistical analysis

Raw sequence data in FASTQ format (16S and ITS2) were subjected to quality control analysis with FastQC software and prepared for taxonomic classification using the Mothur software (version 1.43.0) (Schloss et al., 2009) and following the standard operating protocol proposed by (Kozich et al., 2013). Overlapping pairs of sequence reads were merged into contigs. In addition, reads with ambiguous bases, duplicated contigs and homopolymers longer than 13 bp were removed. The VSEARCH algorithm (embedded in the Mothur framework) was used to remove chimeras and these were subsequently omitted. The resulting sequences were classified according to the taxonomy into the corresponding Operational Taxonomic Units at 97 % similarity, besides using the reference trainset 16_022016.pds from https://mothur.org/wiki/RDP_reference_files for Bacteria and ITS sequences provided by the UNITE ITS database (version 7.2) at <https://unite.ut.ee/repository.php> for Fungi. Undesired lineages such as Plantae, Animalia, Protista, “unknown” and other were removed. The final sequences were then grouped into taxonomic groups (phylum, order, etc), using the phylotype

command in Mothur, which relies upon reference taxonomic outlines to classify sequences to taxonomic bins (Schloss and Westcott, 2011). Relative abundances of different taxonomic levels of each bacterial and fungal group were calculated as the percentage from the total count of reads in each sample using the `get.relabund` command in Mothur. Taxa relative abundances for each study site were calculated by means of the three replicates of each treatment. Phyla (both bacteria and fungi) that showed > 5 % abundance in at least one sampling site and orders that showed > 3 % in at least two sampling sites, were considered.

The sequencing results at order level were used to calculate the Shannon-Weaver index (H') (Shannon and Weaver, 1963) as it follows:

$$H'p = - \sum_{i=1}^S p_i \ln p_i$$

Where p_i is the relative frequency of the order “i” at each sample and S is the number of bacteria or fungi orders at each sample.

Statistical analyses were performed with the software IBM SPSS Statistics 24. Homogeneity of variances was tested using the Levene's test and data were transformed as needed to fit to a normal distribution. The ANOVA of repeated measures was performed in order to evaluate differences along time (within subject factor) and among treatments (between subject factor). Multiple comparisons with Bonferroni test were applied for evaluating the whole effect of the treatments, while pairwise comparisons with Bonferroni test were performed at specific sampling times. The bacterial and fungal relative abundance percentages were analysed by Canonical Correspondence Analysis (CCA) for evaluating the relationship between soil fertility and microbiological groups. The CANOCO software for Windows v4.02 was used for CCA (Ter Braak and Smilauer, 1999).

6.3. Results

6.3.1. Changes of edaphic parameters throughout treatments and samplings

The evolution of the analysed soil parameters along the experiment is shown in Figure 6.3 and Figure 6.4. The *time* factor caused a significant effect in pH ($p < 0.05$) but there was not effect of the treatments on pH variation along time (*time*treatment* factor, $p > 0.05$). Initially, the treatments without biochar, S and SC, showed lower pH values (pH ~ 7.7 , $p < 0.05$, Figure 6.3 A) than those measured at the biochar containing treatments, SB and SBC (pH ~ 7.9 -8.0). However, after three months, all the treatments showed similar pH values (7.4-7.7, $p > 0.05$). In contrast to pH values, the *time* or *time*treatment* factors did not affect significantly the EC values ($p > 0.05$). At any sampling point, treatments containing compost (SC and SCB) showed higher EC values (~ 2.5 dS m^{-1} , $p < 0.05$, Figure 6.3 B) than the other two treatments (S and SB, ~ 2.1 dS m^{-1}).

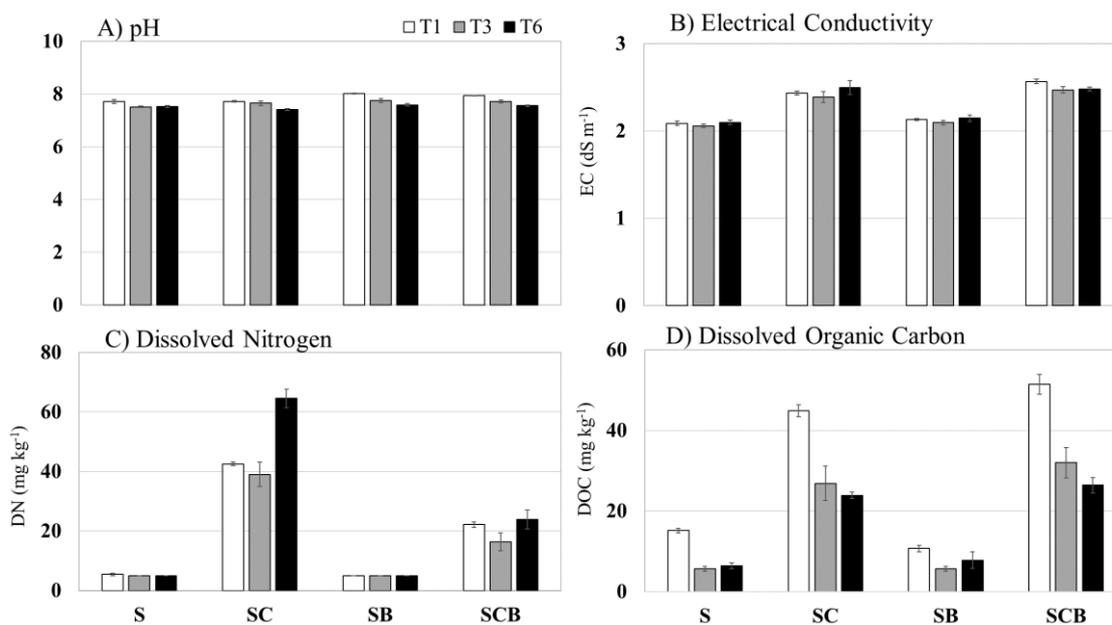


Figure 6.3: Results of the edaphic parameters analysed in the 1:2.5 soil water extract in each treatment (S, SC, SB and SCB) at each sampling time (T1, T3 and T6): pH, electrical conductivity, dissolved nitrogen and dissolved organic carbon. Bars on columns indicate standard error (n=3). The treatments are: S, bulk tailings; SC, tailings + 4 % compost; SB, tailings + 4 % biochar; SCB, tailings + 4 % compost + 4 % biochar.

Similar to EC values, the water extractable K^+ , Ca^{2+} and SO_4^{2-} concentrations (Figure 6.4 A, B and D) were not affected by the *time* or *time*treatment* factors. All the treatments showed similar SO_4^{2-} and Ca^{2+} concentrations (4000-4300 $mg\ kg^{-1}$ SO_4^{2-} and $\sim 2000\ mg\ kg^{-1}$ Ca^{2+}), in contrast to K^+ , whose concentrations were different among treatments ($SBC > SC > SB > S$, $p < 0.05$) at each sampling time. The Cl^- and Mg^{2+} concentrations showed a significant effect of the *time* factor but not of its interaction *time*treatment*. The Cl^- concentrations of the treatments without compost were below the detection limit ($12\ mg\ kg^{-1}$), while those containing compost showed concentrations between 70-200 $mg\ kg^{-1}$ (Figure 6.4 C). For Mg^{2+} , the concentrations in the treatments containing compost, SC and SCB, were at least 1.2-fold higher ($p < 0.05$) than those obtained for the S and SB treatments (Figure 6.4 E). The *time* and *time*treatment* factors showed a significant effect ($p < 0.05$) on the DN and DOC parameters. At the three sampling points, the treatments containing compost (SC and SCB) showed higher DN and DOC concentrations (~ 4 -fold higher values, $p < 0.05$) than the other two treatments (S and SB, Figure 6.3 C and D).

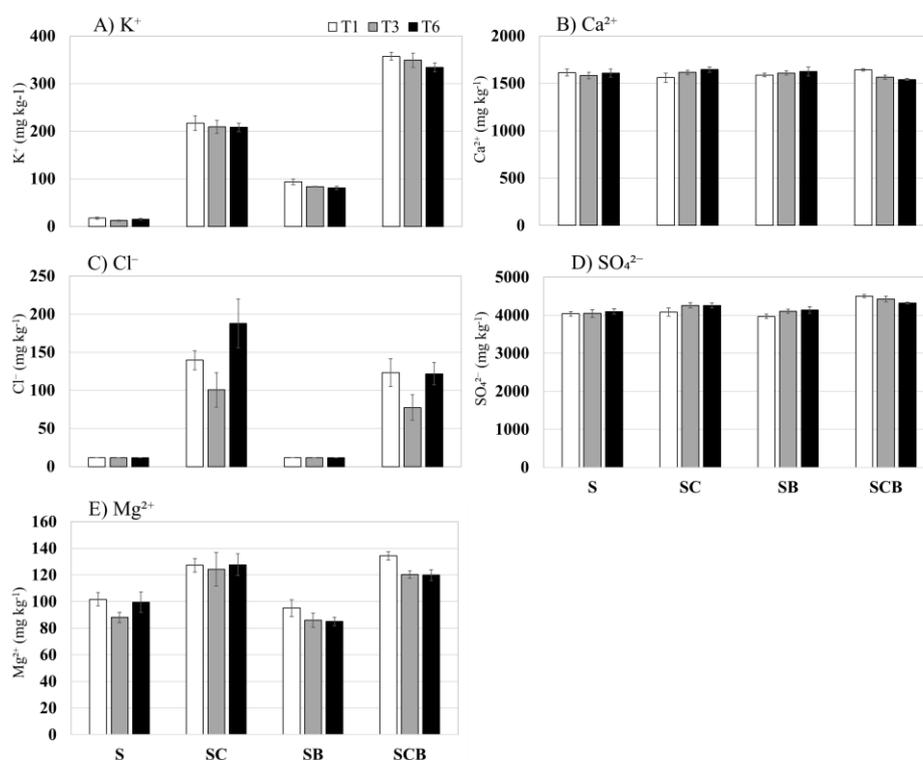


Figure 6.4: Ion concentrations (K^+ , Ca^{2+} , Cl^- , SO_4^{2-} and Mg^{2+}) measured of the 1:2.5 soil:water extract at each treatment (S, SC, SB and SCB) and sampling time (T1, T3 and T6). Bars on columns indicate standard error ($n=3$). The treatments are: S, bulk tailings; SC, tailings + 4 % compost; SB, tailings + 4 % biochar; SCB, tailings + 4 % compost + 4 % biochar.

Lead water extractable concentrations were below the detection limit along the experiment for the four treatments ($< 10 \mu\text{g kg}^{-1}$). The *time* factor significantly affected ($p < 0.05$) the Cu, Mn and Zn concentrations. The effect of the interaction *time*treatment* was only significant for Mn ($p < 0.05$). For Cu (Figure 6.5 A), the treatments containing compost (SC and SCB) showed at least 1.5-fold higher concentrations ($p < 0.05$) than the other two treatments (S and SB) except at the T6, where S and SB concentrations came closer to SC ($p > 0.05$). After one month from the beginning of the experiment (T1 samples), the treatments S and SB showed significant lower Mn concentrations (at least 3-fold lower values, $p < 0.05$, Figure 6.5 B) than those treatments containing compost (SC and SCB). These differences among treatments were not detected along the whole experiment (T3, T6, $p > 0.05$), due to the decrease of Mn concentrations ($p < 0.05$) in the treatments containing compost (SC and SCB). Regarding Zn (Figure 6.5 C), the T1 samples, showed similar Zn concentrations $210\text{-}266 \mu\text{g kg}^{-1}$ ($p < 0.05$) in all the treatments. Nevertheless, at T3 and T6, Zn concentrations decreased significantly ($p < 0.05$) in the treatments containing biochar (SB and SBC, $100\text{-}120 \mu\text{g kg}^{-1}$) in comparison to the other treatments (S and SC, $\sim 200 \mu\text{g kg}^{-1}$).

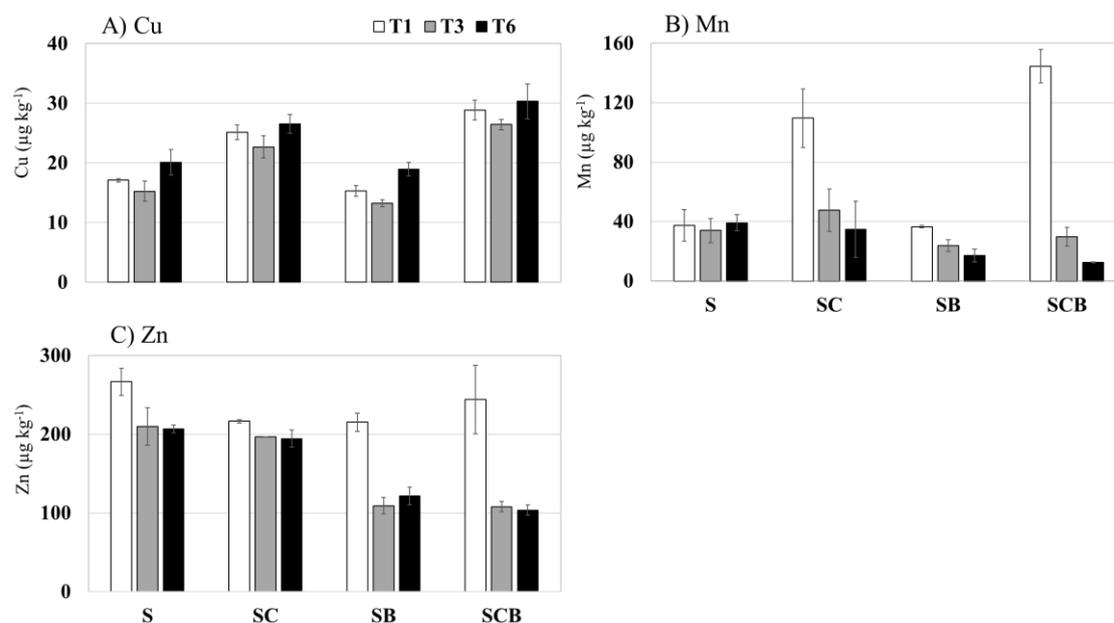


Figure 6.5: Metal concentrations (Cu, Mn and Zn) analysed in the 1:2.5 soil water extract in each treatment (S, SC, SB and SCB) at each sampling time (T1, T3 and T6). Bars on columns indicate standard error (n=3). The treatments are: S, bulk tailings; SC, tailings + 4 % compost; SB, tailings + 4 % biochar; SCB, tailings + 4 % compost + 4 % biochar.

6.3.2. Shannon diversity index for bacteria and fungi

The *time* and *time*treatment* factors caused a significant effect ($p < 0.05$, ANOVA of repeated measures) in the values of the Shannon diversity index for the bacterial orders (Figure 6.6 A). At the initial sampling (T1), the diversity index of the SB treatment showed significant lower values ($p < 0.05$) than the rest of the treatments (S, SC and SCB, $p > 0.05$). However, the SB treatment showed a significant increase along the experiment reaching similar diversity values ($p > 0.05$) to those obtained for the S treatment at the T6 samples. The treatments containing compost (SC and SCB) showed higher diversity ($p < 0.05$) by the end of the experiment (T6 samples) than the other two treatments (S and SB, Figure 6.6).

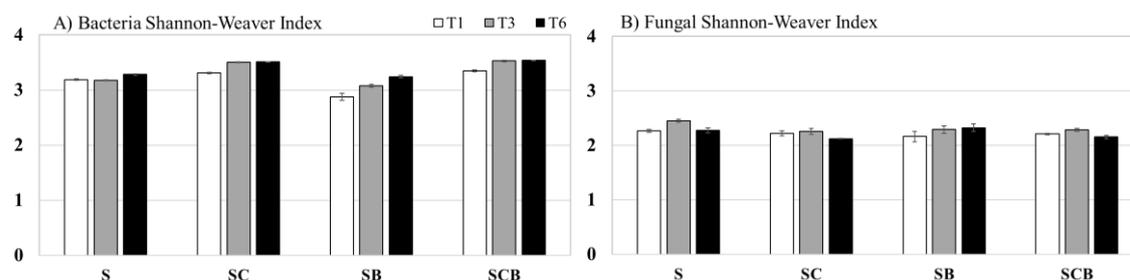


Figure 6.6: Shannon-Weaver index for bacteria (A) and fungi (B) in each treatment (S, SC, SB and SCB) at each sampling time (T1, T3 and T6). Bars on columns indicate standard error (n=3). The treatments are: S, bulk tailings; SC, tailings + 4 % compost; SB, tailings + 4 % biochar; SCB, tailings + 4 % compost + 4 % biochar.

In relation to fungi (Figure 6.6 B), the modifications of the Shannon index values were significantly affected by the *time* factor ($p < 0.05$, ANOVA of repeated measures) but not by the *time*treatment* interaction ($p > 0.05$). Unlike bacteria, fungal diversity did not show differences among treatments at any sampling time ($p > 0.05$).

6.3.3. Influence of treatments on bacterial composition

Bacterial groups belonging to *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, *Planctomycetes* and *Gemmatimonadetes* phyla were the most abundant in our study (Figure 6.7). There was a significant effect of the *time* factor in the variations of relative abundance percentages for all the bacterial phyla ($p < 0.05$, ANOVA of repeated measures). In

addition, the interaction *time*treatment* affected significantly almost all phyla relative abundances ($p < 0.05$), with the exception of the *Actinobacteria* phylum ($p > 0.05$).

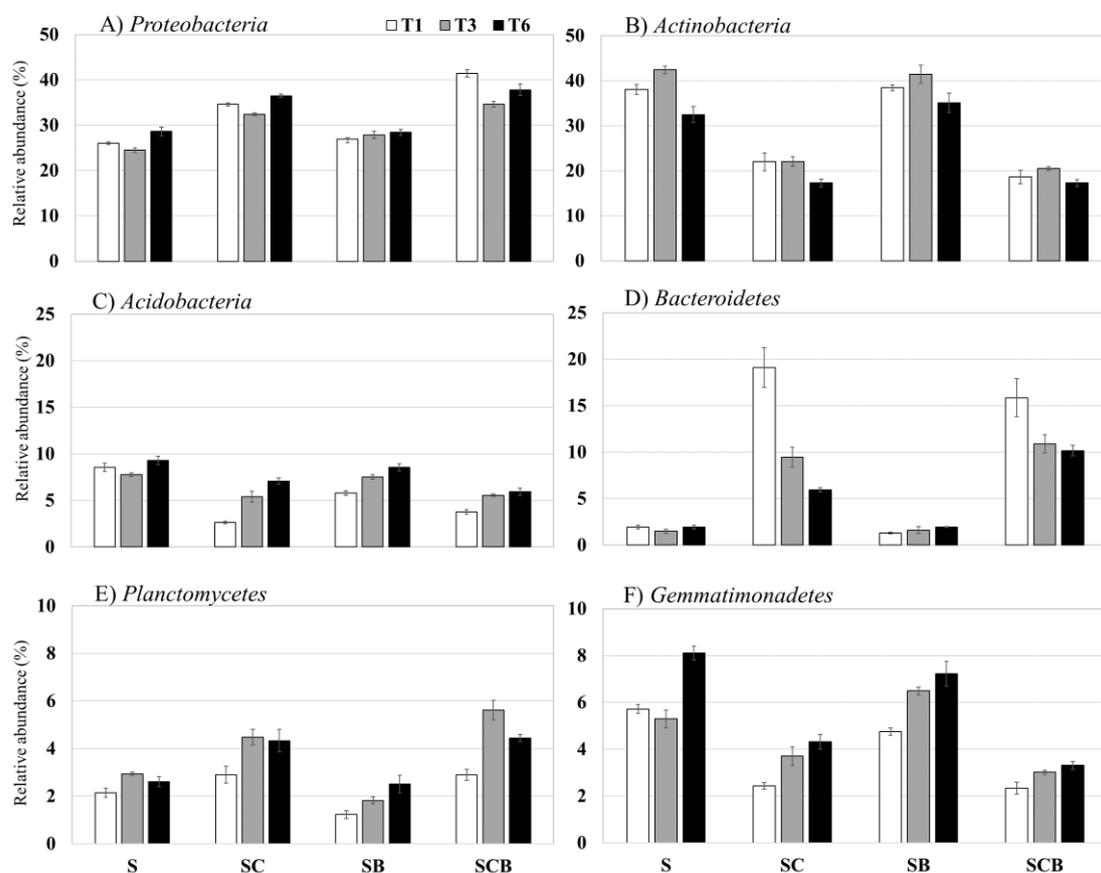


Figure 6.7: Relative abundance percentages of the six main phyla of bacteria (A-F) detected in each treatment (S, SC, SB and SCB) at each sampling time (T1, T3 and T6). Bars on columns indicate standard error (n=3). The treatments are: S, bulk tailings; SC, tailings + 4 % compost; SB, tailings + 4 % biochar; SCB, tailings + 4 % compost + 4 % biochar.

The *Proteobacteria* phylum showed relative abundances higher than 20 % across all the treatments and samplings (Figure 6.7 A). The treatments with compost (SC and SCB) showed higher abundances ($p < 0.05$) than the other two treatments without compost (S and SB). For each treatment, the percentages of *Proteobacteria* abundance at the T6 samples did not differ ($p < 0.05$) from those obtained at the first sampling time (T1). The main orders which contribute to this phylum included *Rhizobiales* and *Sphingomonadales* (*Alphaproteobacteria* class), *Betaproteobacteria uncl.* and *Burkholderiales* (*Betaproteobacteria* class), *Myxococcales* (*Deltaproteobacteria* class) and *Pseudomonadales*, *Gammaproteobacteria uncl.* and *Xanthomonadales* orders (*Gammaproteobacteria* class) (Figure 6.8). These orders showed a

contrasting variation in their abundances along the experiment: while some orders did not vary their relative abundance (e.g. *Sphingomonadales* and *Rhizobiales*, Figure 6.8 A and C); other groups showed significant decreases or increases of relative abundances (e.g. *Pseudomonadales* and *Burkholderiales* and e.g. *Myxococcales*, respectively, Figure 6.8 B, H and E).

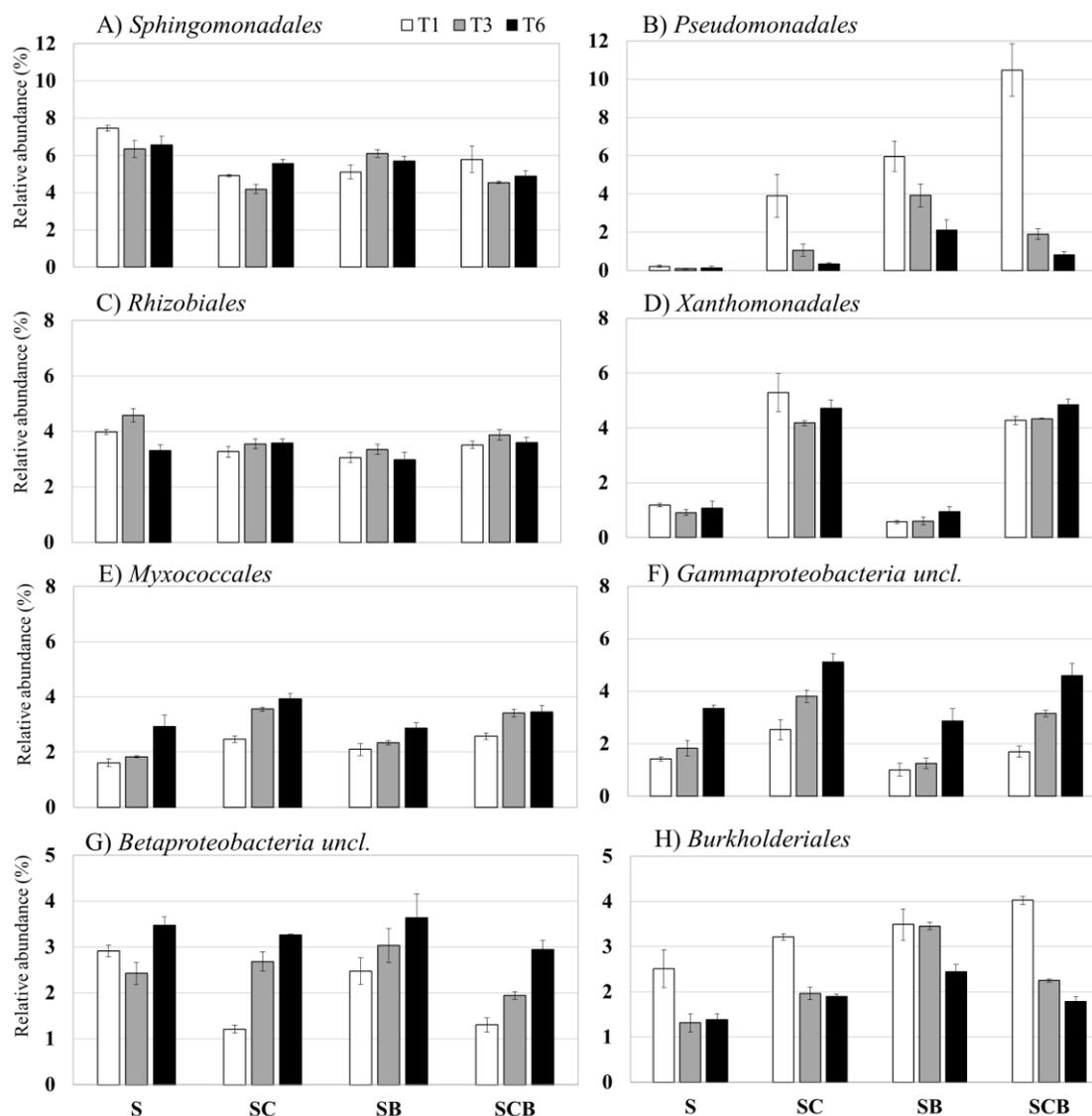


Figure 6.8: Relative abundance of the main bacterial orders of *Proteobacteria* phylum (A-H) in each treatment (S, SC, SB and SCB) at each sampling time (T1, T3 and T6). Bars on columns indicate standard error (n=3). The treatments are: S, bulk tailings; SC, tailings + 4 % compost; SB, tailings + 4 % biochar; SCB, tailings + 4 % compost + 4 % biochar.

Actinobacteria was the only phylum in which no significant ($p > 0.05$) interaction *time***treatment* occurred. In contrast to what occurred for *Proteobacteria*, the treatments without the presence of compost (S and SB) showed higher relative abundance percentages of

Actinobacteria (about double, $p < 0.05$) than those treatments containing compost (SC and SCB, Figure 6.7 B). Three main orders were recorded within this phylum: *Actinomycetales* which decreased its abundance percentage ($p < 0.05$) along the experiment in all treatments (Figure 6.9 B); *Acidimicrobiales* which showed the opposite behaviour, a significant increase of relative abundance ($p < 0.05$) along the experiment in all treatments (Figure 6.9 A); and *Solirubrobacterales* which showed a significant increase ($p < 0.05$) but only in two treatments, SC and SB (Figure 6.9 H).

For the *Acidobacteria* phylum, at the first sampling (T1), the treatments without compost (S and SB) showed higher relative abundances ($p < 0.05$) than the other two treatments (SC and SCB, Figure 6.7 C). Along the experiment (from T1 to T6) there was a significant increase ($p < 0.05$) of the abundance percentages in the three amended treatments (SC, SB and SCB), while the bulk tailings treatment (S) maintained similar data to those obtained at T1. For instance, SC showed three times higher abundance values in T6 in relation to T1. The main identified order corresponded to the Gp7 (Figure 6.9 G).

In the case of the *Bacteroidetes* phylum, the treatments without compost, S and SB, showed lower relative abundance values (at least 10-fold lower, $p < 0.05$) than the treatments containing compost (SC and SCB) along the whole experiment (Figure 6.7 D). There was not variation in the relative abundance values throughout the experiment ($p > 0.05$) in the two treatments without compost (S and SB). However, a significant decrease in the abundance percentages occurred in the two treatments containing compost (SC and SCB) from T1 to the final T6 samples ($p < 0.05$). This tendency was similar for the three main bacterial orders (Figure 6.9 D, E and I) belonging to this phylum: *Sphingobacteriales*, *Flavobacteriales* and *Cytophagales*.

For the *Planctomycetes* phylum (Figure 6.7 E), and its main contributing order *Planctomycetales* (Figure 6.9 F), the relative abundance in the treatments containing compost, SC and SCB, raised along with the experiment, from similar abundance data ($p > 0.05$) to those obtained for the S treatment at T1 to 2-fold higher percentages ($p < 0.05$) than the treatments without compost, S and SB, at T6.

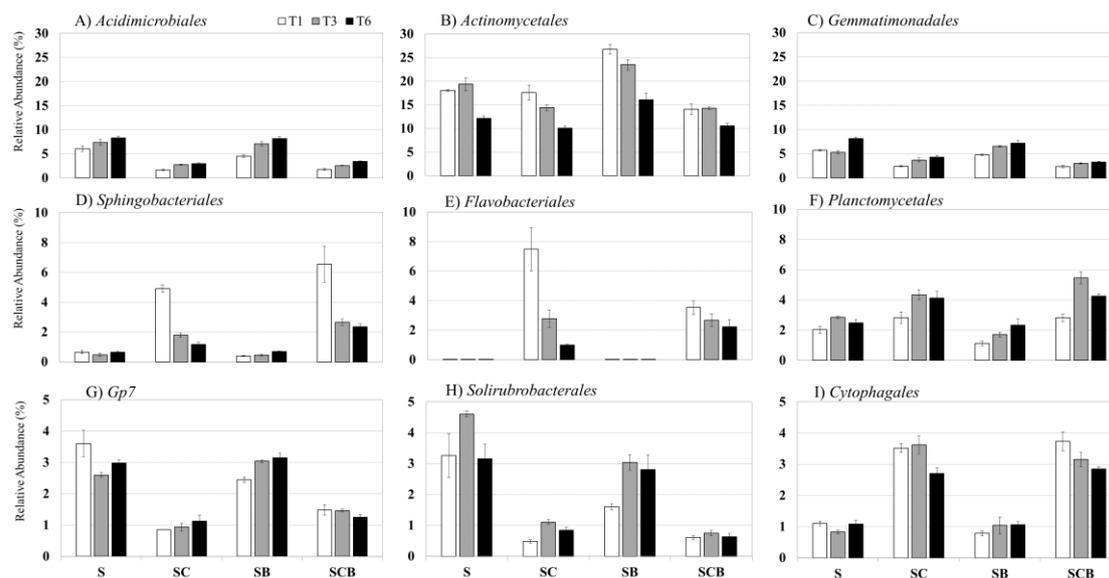


Figure 6.9: Relative abundance of the main bacterial orders of *Actinobacteria* (A, B, H), *Acidobacteria* (G), *Bacteroidetes* (D, E, I), *Planctomycetes* (F) and *Gemmatimonadetes* (C) phyla in each treatment (S, SC, SB and SCB) at each sampling time (T1, T3 and T6). Bars on columns indicate standard error (n=3). The treatments are: S, bulk tailings; SC, tailings + 4% compost; SB, tailings + 4% biochar; SCB, tailings + 4% compost + 4% biochar.

The *Gemmatimonadetes* phylum (Figure 6.7 F), and its order *Gemmatimonadales* (Figure 6.9 C), showed significantly higher abundance percentages ($p < 0.05$) in the treatments without compost (S and SB) than the other two treatments (SC and SCB). There was a significant increase in the relative abundance (about half higher, $p < 0.05$) from T1 to T6 for the S, SC and SB treatments.

Results of the CCA for bacterial orders are shown in the Figure 6.10 and Tables 6.2 and 6.3. Data analyses resulted significant (Monte-Carlo test, $p < 0.05$) and the first axis explained 63.1 % of the variance (Table 6.2). The positive side of the CCA1-axis was defined by increasing concentrations of DOC, DN, extractable elements (K^+ , Cu and Mn) and EC values ($r > 0.64$ for DN, DOC, EC, K^+ , Cu and Mn). In contrast, the negative side of the CCA1-axis was conditioned by lower values of the aforementioned parameters. In this way, the CCA1-axis (Figure 6.10) segregated the bacterial orders between those samples coming from the treatments containing compost, SC (rectangles) and SCB (stars), which were mostly depicted on the positive side of

CCA1-axis, and those belonging to the treatments with no addition of compost, S (circles) and SB (diamonds), which were depicted on the negative side of the CCA1-axis.

Table 6.2: Data of the Canonical Correspondence Analysis (CCA) including eigenvalues of the two first axis and cumulative percentage of variance of bacteria-soil data interaction. The significance of the first canonical axis (Monte-Carlo test, p-value) is also provided.

Parameter evaluated	Axis	Eigenvalue	% cumulative variance	Significance
Bacterial orders	I	0.141	63.1	0.002
	II	0.060	89.7	

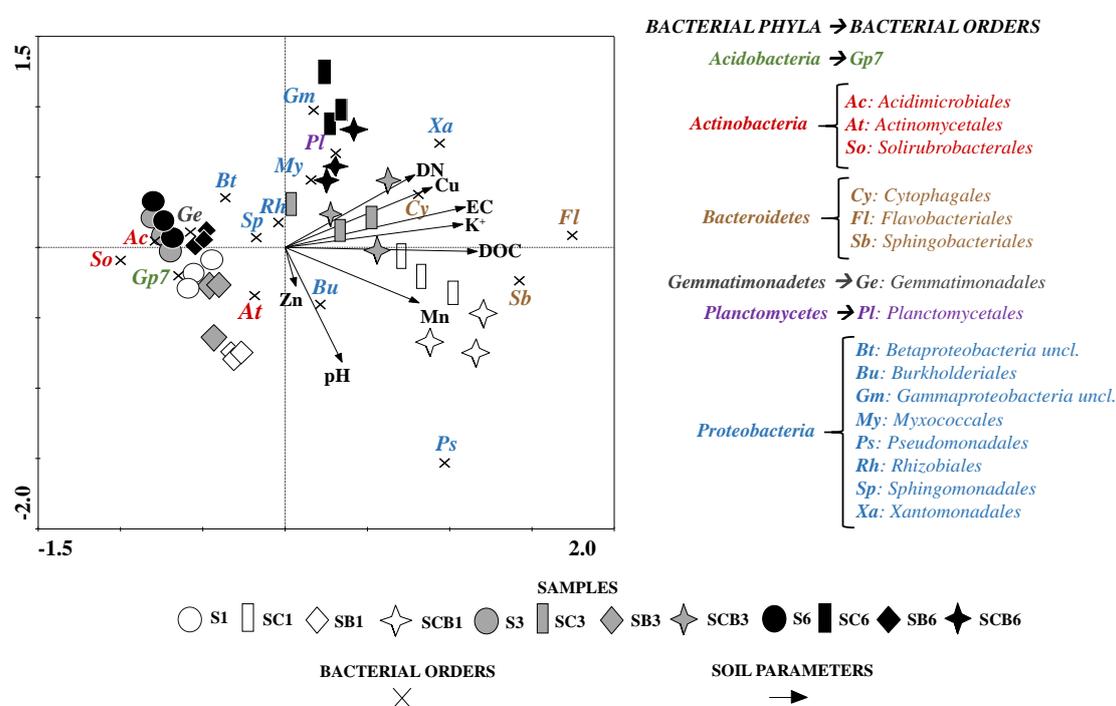


Figure 6.10: Ordination diagram obtained for the Canonical Correspondence Analysis (CCA) of the relative abundance percentages for bacterial orders and selected soil properties in each treatment (S, SC, SB and SCB) at each sampling time (T1, T3 and T6). Cu, Mn, Zn and K⁺ represent water extractable concentrations; “DOC” is Dissolved Organic Carbon concentration; “DN” is Dissolved Nitrogen concentration; “EC” and “pH” are Electrical Conductivity and pH, respectively. All soil parameters were analysed in the 1:2.5 soil:water extract (n=3). Samples from the T1 are represented in white colour; samples from the T3 are represented in grey colour; samples from the T6 are represented in black colour. The treatments are: S, bulk tailings (circles); SC, tailings + 4% compost (rectangles); SB, tailings + 4% biochar (diamonds); SCB, tailings + 4% compost + 4% biochar (stars).

The CCA2-axis (Figure 6.10 and Tables 6.2 and 6.3) was mainly defined by pH ($r = -0.76$) and segregated the samples attending to the sampling time (T1, T3 and T6). For instance, all samples from T1 (white symbols) were depicted on the negative side of the CCA2-axis, while the samples of T6 (black symbols) were depicted on the positive side of the CCA2-axis. This segregation was higher for the treatments containing compost (SC and SCB) and the single biochar (SB) treatment than for the bulk tailings treatment (S).

Table 6.3: Weighted correlation matrix for the first two species axes and environmental variables for bacterial orders.

Soil parameters	Bacterial orders		
	Species Ax1	Species Ax2	
pH	0.28	-0.76	
Electrical Conductivity (EC)	0.88	0.27	
Dissolved Organic Carbon (DOC)	0.94	-0.03	
Dissolved Nitrogen (DN)	0.64	0.48	
K⁺ cation	0.87	0.15	
Water extractable metals	Cu	0.72	0.40
	Mn	0.66	-0.37
	Zn	0.05	-0.26

6.3.4. Influence of treatments on fungal composition

Fungal taxa belonging to the *Ascomycota*, *Basidiomycota* and *Chytridiomycota* phyla were detected in the four treatments (Figure 6.11). There was a significant effect of the *time* factor in the variations of relative abundance percentages for the *Basidiomycota* phyla ($p < 0.05$, ANOVA of repeated measures) but not for the other two phyla. The interaction *time*treatment* did not result significant for any phyla ($p > 0.05$, ANOVA of repeated measures).

The *Ascomycota* phylum showed relative abundances higher than 60 % in all treatments at every sampling time (Figure 6.11 A). Significant differences among treatments occurred at the last sampling time (T6), when the SC treatment (~ 63 %) showed lower abundance ($p < 0.05$) than the treatments without compost (S and SB, ~ 80 % abundance). This was mainly due to the contribution of three orders, *Eurotiales*, *Pleosporales* and *Chaetothyriales*, which showed higher

abundances in the treatments without compost (S and SB) than the other two treatments (SCB and SC, Figure 6.12 B, C and G). In contrast, the *Sordariomycetes uncl.*, *Sordariales* and *Microascales* orders, showed at least 2-fold higher relative abundances ($p < 0.05$) at the treatments containing compost than the treatments without compost (S and SB, Figure 6.12 D, E and H). The *Hypocreales* and *Ascomycota uncl.* orders did not show a clear pattern throughout the experiment (Figure 6.12 A and F).

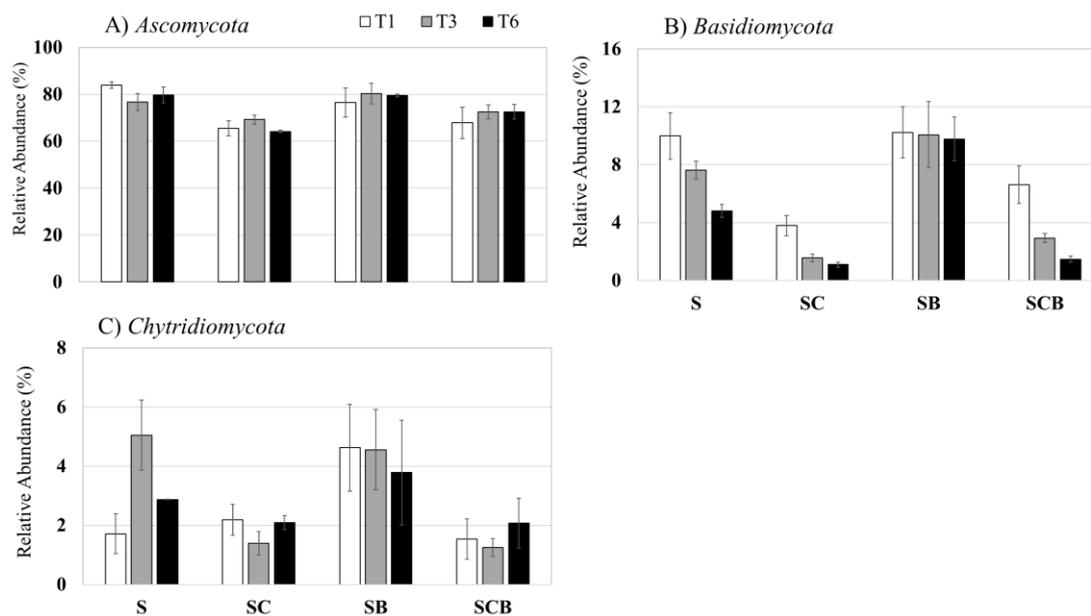


Figure 6.11: Relative abundance of the three main phyla of fungi (A, B and C) detected in each treatment (S, SC, SB and SCB) at each sampling time (T1, T3 and T6). Bars on columns indicate standard error (n=3). The treatments are: S, bulk tailings; SC, tailings + 4 % compost; SB, tailings + 4 % biochar; SCB, tailings + 4 % compost + 4 % biochar.

The *Basidiomycota* phylum showed a decrease of relative abundances along time ($p < 0.05$) in the S, SC and SCB treatments (Figure 6.11 B). By contrast, the abundance percentages of the SB treatment remained unaltered throughout the experiment and they were higher than the rest of the treatments ($\sim 10\%$, $p < 0.05$) at the last sampling time (T6). The main orders identified within this phylum were *Agaricales* and *Thelephorales* (Figure 6.12 I and J). The latter showed higher relative abundances in the S and SB treatments (around 5 %) at the first sampling (T1) compared with the SC and SCB samples. However, they showed a sharp decrease (half of that value) along the experiment. As it occurred for the corresponding phylum data, both *Agaricales*

and *Thelephorales* showed 2-fold higher relative abundance percentages ($p < 0.05$) in the SB treatment than the rest of the treatments.

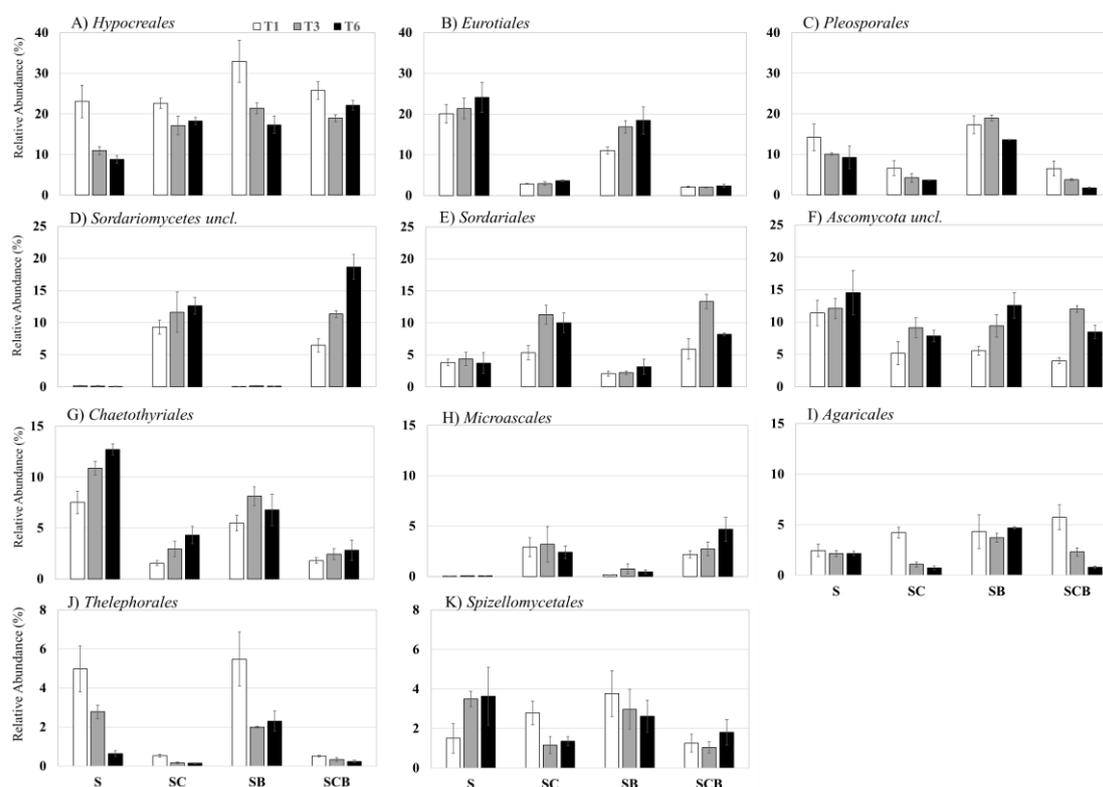


Figure 6.12: Relative abundance percentages of the main fungal orders of *Ascomycota* (A-H), *Basidiomycota* (I, J) and *Chytridiomycota* (K) phyla in each treatment (S, SC, SB and SCB) at each sampling time (T1, T3 and T6). Bars on columns indicate standard error (n=3). The treatments are: S, bulk tailings; SC, tailings + 4 % compost; SB, tailings + 4 % biochar; SCB, tailings + 4 % compost + 4 % biochar.

The *Chytridiomycota* phylum (Figure 6.11 C) was only composed by the *Spizellomycetales* order (Figure 6.12 K). This fungal group did not show significant differences among treatments along the experiment ($p > 0.05$).

Results of the CCA of fungal orders were significant (Monte-Carlo test, $p < 0.05$) and the first axis explained 78.3 % of the variance (Figure 6.13 and Tables 6.4 and 6.5). The positive side of the CCA1-axis was mainly defined by increasing values of EC, fertility parameters (K^+ , DOC and DN), and water extractable Cu concentrations (e.g. $r = 0.90$ for EC and K^+). The samples coming from the treatments containing compost, SC (rectangles) and SCB (stars), were mostly

depicted on the positive side of CCA1-axis, and those belonging to the treatments without compost, S (circles) and SB (diamonds) were depicted on the negative side of the CCA1-axis.

Table 6.4: Data of the Canonical Correspondence Analysis (CCA) including eigenvalues of the two first axis and cumulative percentage of variance of fungal-soil data interaction. The significance of the first canonical axis (Monte-Carlo test, p-value) is also provided.

Parameter evaluated	Axis	Eigenvalue	% cumulative variance	Significance
Fungal orders	I	0.261	78.3	0.002
	II	0.053	94.1	

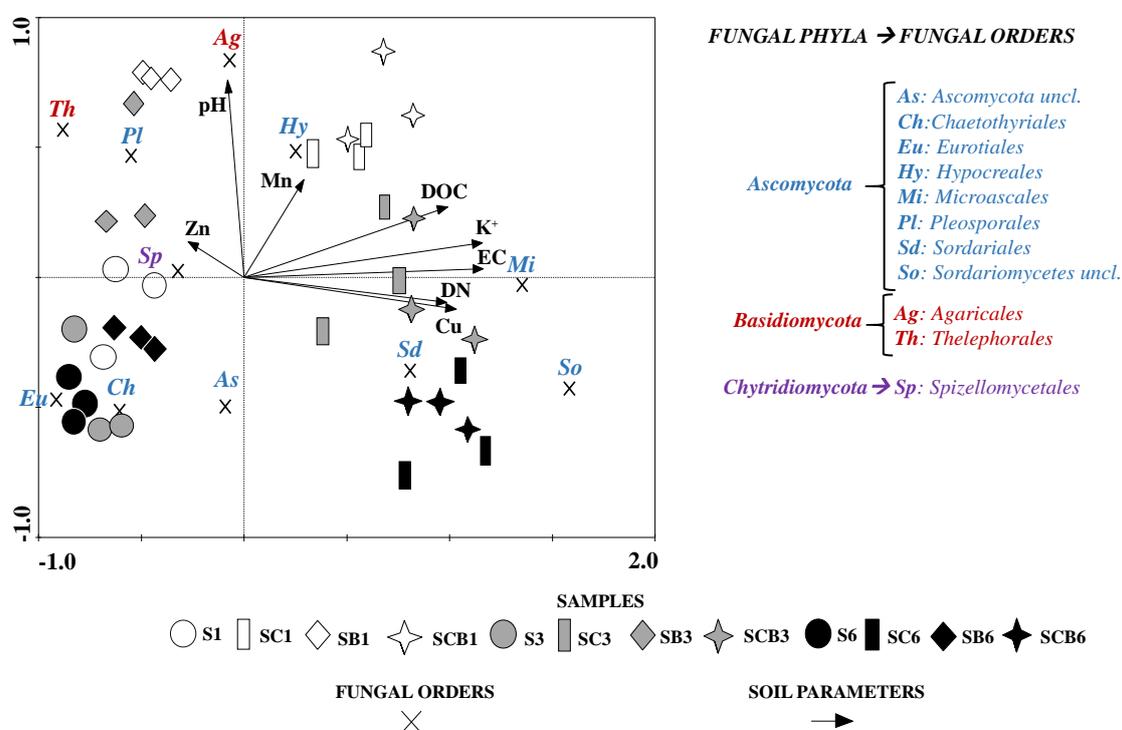


Figure 6.13: Ordination diagram obtained for the Canonical Correspondence Analysis (CCA) of the relative abundance percentages for fungal orders and selected soil properties in each treatment (S, SC, SB and SCB) at each sampling time (T1, T3 and T6). Cu, Mn, Zn and K^+ represent water extractable concentrations; “DOC” is Dissolved Organic Carbon concentration; “DN” is Dissolved Nitrogen concentration; “EC” and “pH” are Electrical Conductivity and pH, respectively. All soil parameters were analysed in the 1:2.5 soil:water extract (n=3). Samples from the T1 are represented in white colour; samples from the T3 are represented in grey colour; samples from the T6 are represented in black colour. The treatments are: S, bulk tailings (circles); SC, tailings + 4 % compost (rectangles); SB, tailings + 4 % biochar (diamonds); SCB, tailings + 4 % compost + 4 % biochar (stars).

On the other hand, the positive side of the CCA2-axis was mainly defined by high pH values ($r = 0.78$) and to a lesser extent by increasing values of Mn concentrations ($r = 0.39$), while the negative side was mainly conditioned by lower values of these two parameters. So, a gradient was generated along the CCA2-axis, which segregated the samples from the amended treatments (SB-diamonds, SC-rectangles and SCB-stars) attending to the sampling times (T1, T3 and T6, Figure 6.13): the samples from T1 were depicted on the positive side of the CCA2-axis, while the corresponding T3 and T6 samples appeared consecutively on the negative side of the CCA2-axis. This segregation was not so evident for the samples of the bulk tailings treatment (circles).

Table 6.5: Weighted correlation matrix for the first two species axes and environmental variables for fungal orders.

Soil parameters		Fungal orders	
		Species Ax1	Species Ax2
pH		-0.06	0.78
Electrical Conductivity (EC)		0.90	0.03
Dissolved Organic Carbon (DOC)		0.77	0.28
Dissolved Nitrogen (DN)		0.76	-0.10
K⁺ cation		0.90	0.14
Water extractable metals	Cu	0.80	-0.13
	Mn	0.23	0.39
	Zn	-0.21	0.14

6.4. Discussion

6.4.1. Effects of amendments on soil parameters

Soil microbial composition can be easily modified by the addition of amendments such as compost or biochar (Abujabhah et al., 2016). The extent of these changes depends on soil properties such as pH, moisture and especially, organic carbon (Zornoza et al., 2015; Asemaninejad et al., 2021). In our experiment, the addition of compost caused stronger effects in most of soil parameters than the biochar, especially in the SC treatment (Figure 6.3, 6.4 and 6.5). This might be explained by the larger pool of readily available compounds released by the compost, especially at the T1. After this initial flush, there was a gradual decrease in the DOC

and/or extractable Mn and Zn concentrations, probably because the labile pools became shorter with the time (Párraga-Aguado et al., 2015). In contrast, the higher recalcitrant organic matter content (low labile pool and higher stability) and lower metal load of the biochar in relation to the compost conditioned lower modifications in those parameters at the SB treatment (Rodríguez-Vila et al., 2016; You et al., 2018). In the treatment combining biochar-compost, most of the studied parameters were mainly influenced by compost (e.g. DOC) and results differed from those at the single composted treatment in the DN (that was lower) and K⁺ (that was higher) values. Lower DN concentrations in the combined biochar-compost treatment could be attributed to the retention of water-soluble nitrogen by biochar (Haider et al., 2016).

6.4.2. Effects of amendments on bacterial composition

In terms of phylum abundance, the compost caused a significant increase in the percentages of *Proteobacteria* and *Bacteroidetes* (Figure 6.7). Similar increases in the relative abundance of these two phyla were also reported by Cao et al. (2020) in compost amended coal mining soils.

The controlled conditions of the growth chamber (temperature, light and humidity) might influence the relative abundances observed for several bacterial groups along the experiment. This could explain why several bacterial orders (Figures 6.8 and 6.9) showed similar trends in all treatments (including the bulk tailings, S). This was the case of *Myxococcales*, *Burkholderiales* and *Gammaproteobacteria uncl.* from the *Proteobacteria* phylum, *Acidimicrobiales* and *Actinomycetales* from the *Actinobacteria* phylum and *Gemmatimonadales* from the *Gemmatimonadetes* phylum.

In the initial T1 sampling, the presence of compost showed a stronger effect on soil microbial composition than the biochar. This was clearly shown in Figure 6.10, where the main gradient defined by the CCA1-axis segregated between samples with and without compost. The initial effect of biochar on the bacterial composition in the SB treatment was restricted to a lower number of bacterial orders than the compost, resulting also in a lower microbial diversity index

(Figure 6.6 A). The modifications of bacterial composition promoted by biochar are highly dependent on its type of raw materials and conditions of pyrolysis, which determine the final effects on soils (Abujabhah et al., 2016). In this case, the conditions of production of the biochar at high temperature (900°C) generated a highly stable material with low carbon accessibility for microorganisms (You et al., 2018). The biochar only caused a significant increase of the relative abundance of the *Actinomycetales* order, belonging to the *Actinobacteria* phylum. This could be due to the specific ability of *Actinomycetales* taxa for degrading hard to decompose organic materials (Tian et al., 2014; Bhatti et al., 2017) and dealing with high concentrations of inorganic contaminants (Risueño et al., 2020a, Chapter 4), which could have provided a competitive advantage in relation to the rest of bacteria.

Initially (at T1), the treatments containing compost showed a bacterial composition characterized by orders which are related to the degradation of organic matter (Figures 6.7, 6.8 and 6.9), such as *Sphingobacteriales*, *Flavobacteriales* and *Cytophagales* from the *Bacteroidetes* phylum (Fierer et al., 2007), *Xanthomonadales*, *Pseudomonadales*, *Burkholderiales* and *Gammaproteobacteria uncl* from the *Proteobacteria* phylum (Kerstens et al., 2006; Tang et al., 2019) or *Planctomycetales* from the *Planctomycetes* phylum (Wiegand et al., 2018). However, some of these orders such as *Flavobacteriales*, *Sphingobacteriales*, *Pseudomonadales*, and *Burkholderiales* showed a decrease of their abundance percentages throughout the experiment. The reasons of this drop might include a toxic effect of the tailings edaphic properties (e.g. salinity, metals) and/or a correlation ($r > 0.5$, $p < 0.01$) with the decreasing concentrations of dissolved organic carbon, which serves as a source of energy. Several studies have shown the relevance of the dynamics of soil organic matter as a critical driver, which determines the shifts in microbial composition during the reclamation of mining soils (Zornoza et al., 2015; Asemaninejad et al., 2021). For instance, most taxa included in the *Bacteroidetes* phylum are considered copiotrophic and their relative abundances are highly dependent of the availability of labile carbon (Fierer et al., 2007). In our study, the relative abundances of the orders belonging to the *Bacteroidetes* phylum, *Flavobacteriales*, *Sphingobacteriales* and *Cytophagales*, were

positively correlated with the decreasing DOC concentrations ($r > 0.8$, $p < 0.01$). In the case of the *Cytophagales* order, the decrease of the abundance percentages along the time was less sharp, probably because of its less dependence on labile organic matter and better adaptation to degrade more complex organic compounds (Kirchman, 2002; Reichenbach, 2006).

Several bacterial taxa maintained the initial increase of abundance percentages promoted by the compost addition, showing certain level of adaptation to the tailings properties and decreasing DOC concentrations throughout the experiment. This group includes the *Proteobacteria* orders belonging to the *Gammaproteobacteria* class, *Xanthomonadales*, and *Gammaproteobacteria uncl.*, which have been observed as representative of the microbiome in mature edaphic successional stages of reclaimed mine tailings (Colin et al., 2019). Some orders belonging to *Gammaproteobacteria* class are also known by their lithotrophic nature (Wakelin et al., 2012) and their capacity for adaptation to extreme soil conditions such as low nutrient concentrations or high salinity, including barren tailings materials (Sun et al., 2018). The high abundances of these *Gammaproteobacteria*'s orders can be also supported by their well-known capacity of dealing with high SO_4^{2-} soil concentrations (Shimkets et al., 2006; Edwardson and Hollibaugh, 2017) for what they showed a significant correlation ($r > 0.6$, $p < 0.01$). The *Myxococcales* order belonging to the *Deltaproteobacteria* class showed a stability in its abundance percentages throughout the experiment. This can be explained by their ability to break down a high variety of organic compounds including those considered as hard to decompose (Shimkets et al., 2006), which could result in a lower dependence on labile organic matter (no significant correlation between abundance percentages and DOC). The *Planctomycetales* order (*Planctomycetes* phylum) showed higher abundances in the T6 than in the T1 samples of the treatments containing compost. This may be due to its lower competitive capacity within nutrient rich media (T1) (Burgess et al., 2020) and its ability to decompose more complex organic matter compounds (Probandt et al., 2017; Wiegand et al., 2018), as it was expected to occur by the end of the experiment, at T6.

The abundance of the *Proteobacteria* orders, *Rhizobiales* and *Sphingomonadales*, were unaffected by the amendments. The bacterial taxa belonging to the *Rhizobiales* order play an important role in the dynamics of N in the soil (e.g. N₂ fixation) but their activity is strongly dependent on the presence of plants (Burges et al., 2020). The *Sphingomonadales* order is also related to plant rhizospheres and has been previously reported as a plant growth promoting bacteria in extreme environmental conditions including mine tailings (Risueño et al., 2020b, Chapter 5) or saline soils (Oliveira et al., 2014). That strong dependence on plant rhizospheres of these two orders may explain the no variation of their abundance percentages during the experiment.

A group of bacterial orders was negatively impacted by the addition of compost. These orders include oligotrophic bacteria whose abundance is favoured in extreme environments but negatively affected in nutrient rich systems, where fast-growing bacteria show a better competitive behaviour (Burges et al., 2020). Similarly to what occurred at our experiment, Wang et al. (2017) showed a decrease in the relative abundance of *Gp7* when adding organic fertilizers into an orchard soil, while Burges et al. (2020) observed a decrease of *Gemmatimonadales* abundance percentages after adding compost in a metal polluted soil. Risueño et al. (2020a, Chapter 4) reported *Acidimicrobiales* as one of the most abundant orders in bulk tailings areas but it was displaced by other organotrophic bacteria in the presence of vegetation.

6.4.3. Effects of amendments on fungal composition

Several studies on the reclamation of polluted sites have shown *Ascomycota* and *Basidiomycota* as the most abundant phyla in these systems (Ma et al., 2013; Bastida et al., 2017). The *Ascomycota* phylum is known to be the largest fungal group in ecosystems and includes from saprotrophs, necrotrophic or biotrophic parasites of plants and animals to endophytes or mutualistic symbionts (Webster and Weber, 2007). This wide range of life conditions for the taxa of this phylum allows their adaptation to several polluted environments such as mine impacted areas (Op De Beeck et al., 2015; Narendrula-Kotha and Nkongolo, 2017; Rosenfeld et al., 2018).

On the other hand, the taxa belonging to the *Basidiomycota* phylum are more abundant in non-polluted areas or associated to vegetation in restored areas (Op De Beeck et al., 2015), probably because of their more specific lifestyles as ectomycorrhizal fungi or saprotrophs (Webster and Weber, 2007). In addition, the saprophytic fungal taxa of the *Basidiomycota* phylum are involved in the decomposition of recalcitrant organic materials, which normally cannot be accessed by those taxa of the *Ascomycota* phylum (Lundell et al., 2010). Finally, the *Chytridiomycota* phylum was also identified although in lower abundance percentages (Figure 6.11 C). The taxa from this phylum can also colonize a wide variety of environments, being considered the order identified in this study, *Spizellomycetales*, of saprotrophic behaviour (Webster and Weber, 2007).

Similar to what occurred with bacteria, the addition of compost showed a stronger effect on fungal relative abundance than the biochar. This was clearly shown in Figure 6.13, where the main gradient defined by the CCA1-axis segregated the samples with and without compost. However, unlike bacteria, only a few orders showed an increase of the relative abundances in the treatments containing compost (*Sordariomycetes uncl.*, *Sordariales* and *Microascales*), while the rest showed a decrease (*Eurotiales*, *Pleosporales*, *Chaetothyriales* and *Telephorales*) or no clear effect (*Hypocreales*, *Ascomycota uncl.* *Agaricales* and *Spizellomycetales*). Similar to what occurred in our experiment, Siles et al. (2014) reported an increase in the abundance percentages of *Sordariomycetes uncl.* and *Sordariales* and a decrease of *Eurotiales* and *Pleosporales* in a pot trial which used olive residues as an amendment. These authors explained the contrasting behaviour of these fungal orders by their response to specific compounds contained in the amendment, whether beneficial or toxic. In other fungal orders, such as the case of *Telephorales* the decrease in the abundance percentages due to compost could be related to a lower competitive behaviour in relation to other fungal taxa (Courty et al., 2010). This revealed the strong relationship between fungal groups and the characteristics of the C source, which can strongly shape fungal composition (Bastida et al., 2013, 2015). In addition, the treatments containing compost showed significant higher electrical conductivity (Figure 6.3 B), which may have also a strong effect on selecting those salinity tolerant taxa (Zeng et al., 2020).

No significant effects of biochar on abundance percentages were found for any order. Several authors have reported no effects (Elzobair et al., 2016) or even a depletion of fungal activity (Warnock et al., 2010; Liao et al., 2016) after applying different types of biochar in agricultural soils. In spite on these results, other studies have determined substantial positive effects on the application of biochar on fungal growth (e.g. Warnock et al., 2007; Abujabhah et al., 2016). These discrepancy of results reveal the importance of the biochar properties (nature, C:N ratio, temperature during pyrolysis) in the final fungal taxa composition (Elzobair et al., 2016) along with the necessity of performing controlled comparative experiments to elucidate the feasibility of the organic amendments in the enhancement of microbiological activity.

6.5. Conclusions

The results derived from the application of two organic amendments, manure compost and biochar, and their combination revealed contrasting effects of each type of amendment on some edaphic parameters and in turn, on microbial composition. While the presence of compost determined the occurrence of microbial groups with strong dependence on labile carbon, biochar favoured decomposers specialized in high lignin substrates or other recalcitrant carbon compounds. The combined treatment biochar-compost may result the best option to support a more diverse microbial population in terms of soil functionality that is able to decompose both labile and recalcitrant carbon compounds. This may favour the resilience of the system against environmental stressors. These conditions may allow the development of some microbial groups which also play an important role on plant establishment. Future research should focus on evaluating how the microbial community resulted from amended tailings is able to fit within the rhizospheric microbiome of the plant species employed in the phytomanagement of these systems.

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CHAPTER 7. Can *Piptatherum miliaceum* or its litter shape the microbial composition in a mine tailings soil previously amended with biochar and/or manure compost?

Can *Piptatherum miliaceum* or its litter shape the microbial composition in a mine tailings soil previously amended with biochar and/or manure compost?

Abstract

As it was concluded in the previous chapter (Chapter 6), the use of organic amendments for the phytostabilization of mine tailings, favoured at short term (right after being applied) the development of some microbial groups implicated in the degradation of organic substrates and plant establishment. However, after six months of incubation some of them were not able to keep up when labile organic carbon concentrations decreased. The goal of this Chapter was to evaluate the effect of the presence of plants or plant litter on the microbial composition in the previously amended tailings (with biochar and/or manure compost). This would allow to determine the feasibility of amendments for the long-term phytomanagement, as well as the ability or inability of the microbial community resulted from the amended tailings to support biogeochemical cycles without additional external inputs of amendments compatible with the rhizospheric microbiome or litter. For this purpose, a pioneer plant species (*Piptatherum miliaceum*) of the former Cartagena-La Unión Mining District tailings was selected. *Piptatherum miliaceum* seeds or its litter were incorporated to previously amended tailings substrates. Our experiment showed that the effects of plant and litter in microbial composition was dependent on the previous presence of amendments, especially compost. At those treatments containing compost, the higher growth of plants was able to shape microbial composition (both, bacterial and fungi), while at the non-amended and biochar-only containing treatments (where a scarce growth of plants occurred), litter showed a more important role, especially in shaping bacterial composition. The higher growth of plants favoured some bacterial groups, whose relative abundances were previously depleted in the compost containing treatments (e.g. *Actinomycetales*) and other groups related to plant rhizospheres (e.g. *Rhizobiales*). These results revealed the ability of plants for shaping their own rhizospheric microbiome when the amendments contribute to ameliorate the low fertility of mine tailing soils. The employment of the combinations biochar-compost could result optimal to achieve the establishment of a vegetation cover at tailings, providing at the same time an effective tool to stimulate plant growth (higher plant biomass), a recalcitrant source of organic matter (biochar) and a support for the development of a plant rhizospheric microbiome.

7.1. Introduction

Mine tailings are structures formed by the accumulation of the left-over materials coming from the refining process of mine ores. Tailings properties include extreme pHs, high salinity, high metal(loid) concentrations and/or low fertility, which provide unsuitable conditions for plant growth (Párraga-Aguado et al., 2013). Abandoned mine tailings piles are of critical environmental concern because erosion can favour the spread of metal(loid) enriched particles from their bare surfaces (Conesa and Schulin, 2010). The phytomanagement by phytostabilization of mine tailings is considered a suitable tool to reduce the environmental risks associated to this specific type of mining wastes (Robinson et al., 2009). Those techniques are based on the improvement of a self-sustaining vegetation cover that protects the soil against wind and water erosion (Robinson et al., 2009; Huang et al., 2011). However, the successful establishment of plants at tailings requires a previous amelioration of edaphic constraints, which can be easily achieved by the incorporation of organic amendments (Clemente et al., 2010; Párraga-Aguado et al., 2015). Organic amendments may contribute to plant establishment by improving soil fertility, providing nutrients for plants (Clemente et al., 2012; Párraga-Aguado et al., 2015) and stimulating soil microbiological activity (Rodríguez-Berbel et al., 2020; Wang et al., 2021). Recent works on the phytomanagement of mine tailings have pointed out the critical role of soil microbiology for the successful establishment of vegetation (Kolaříková et al., 2017; Thavamani et al., 2017; Sun et al., 2018). Soil microorganisms may interact with plant rhizospheres, acting as filters to decrease the phytotoxic effects of metal(loid)s and as supporters of C-N cycles providing available nutrients for plant growth (Thavamani et al., 2017).

Our previous work, shown in the Chapter 6, focused on the effects in microbiological successional processes coming from the addition of organic amendments (manure compost and/or biochar). That assessment results critical because the autochthonous microbiology present at tailings, and already adapted to the specific soil constraints, might be negatively impacted by those microorganisms contained in the amendments or by the changes in soil conditions that amendments may generate (Grandlic et al., 2008). The results of Chapter 6 showed that the use

of organic amendments (compost/biochar) favoured the development of some microbial groups implicated in the degradation of complex organic substrates and plant establishment such as *Actinomycetales* or *Burkholderiales*. Then, the key to achieve the long-term sustainability of the system would lay in how the microbial community resulted from the amended tailings is able to support biogeochemical cycles without additional external inputs of amendments (Pepper et al., 2012) and be compatible with the rhizospheric microbiome or litter of those plants employed in the phytomanagement.

The goal of this work was to evaluate the effect of the presence of plants or plant litter on the microbial composition in previously amended tailings (with biochar and/or manure compost). This would allow to determine the feasibility of amendments for the long-term phytomanagement of those tailings. For this purpose, a pioneer plant species of tailings *Piptatherum miliaceum* of the former Cartagena-La Unión Mining District was selected. Its seeds or litter were incorporated to previously incubated amended tailings substrates. A characterisation of soil parameters (pH, electrical conductivity, dissolved organic carbon, dissolved nitrogen and water extractable metals) and soil microbial composition (bacteria and fungi) was carried out after 12 months.

7.2. Material and methods

7.2.1. Experimental set-up

The experimental set-up was developed using the pots described in the Chapter 6 (Figure 7.1). Therefore, the six months period, in which the experiments included in Chapter 6 were performed, were considered as the initial incubation time for this experiment.

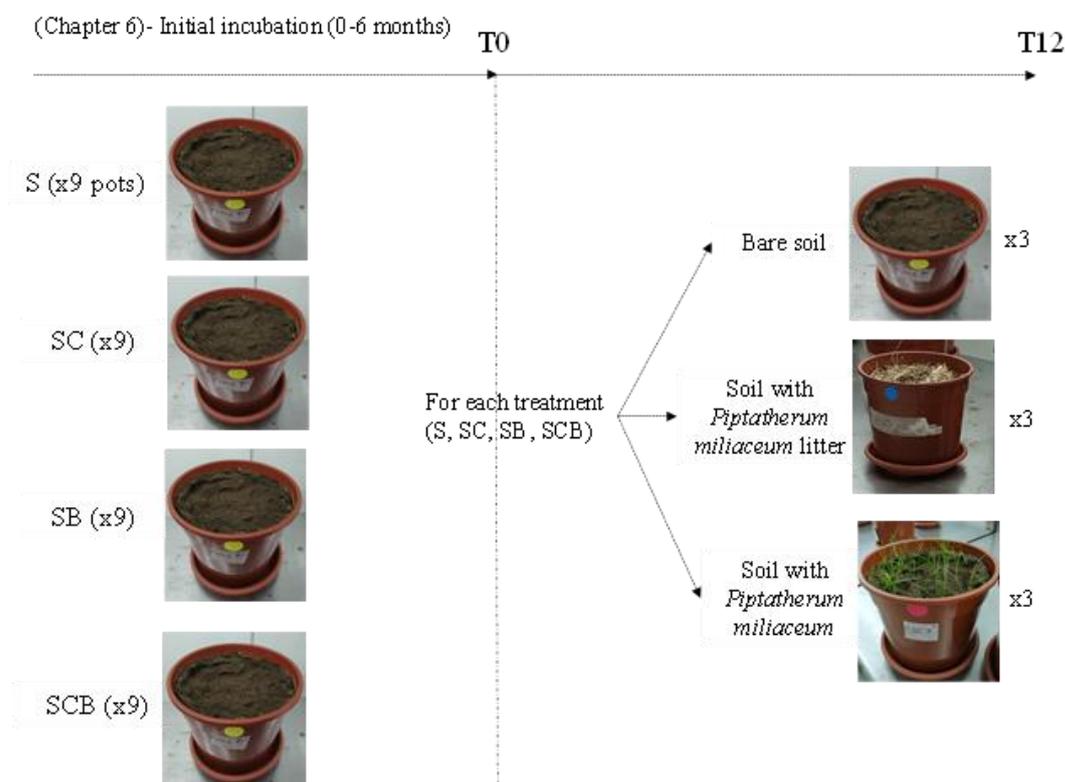


Figure 7.1: Scheme of the treatments carried out. The treatments were: S, bulk tailings; SC, tailings + 4 % compost; SB, tailings + 4 % biochar; SCB, tailings + 4 % compost + 4 % biochar.

For each one of the four treatments considered in that Chapter 6 (S, bulk tailings; SC, bulk tailings + 4 % composted manure; SB, bulk tailings + 4 % biochar; SCB, bulk tailings + 4 % biochar + 4 % composted manure) three new groups of pots were arranged as it follows (Figure 7.1): 1) a group of pots without modification; 2) a group in which plant litter of *Piptatherum miliaceum* was added on top; and 3) a group in which *P. miliaceum* seeds were planted. Each one of these three groups were composed by three replicates.

The litter and seeds used in the experiment were collected from *P. miliaceum* plants, which grew at the mine tailings piles sampled in the field survey included in the Chapters 4 and 5. The selected plant species, *Piptatheim miliaceum* (L.) Coss. subsp. *miliaceum*, has been described as a pioneer plant colonizer in mine tailings (Conesa et al., 2006; Párraga-Aguado et al., 2014). The litter consisted of dry leaves and stalks, which were cut into small pieces (≤ 5 cm) and homogenized. Then, they were washed carefully with distilled water and dried in an oven at 70°C for 72 hours. The litter was characterized by 44 % of total carbon and 0.64 % of nitrogen

and contained metal concentrations of 22 mg kg⁻¹ Zn, 38 mg kg⁻¹ Mn, 5 mg kg⁻¹ Cu or 2 mg kg⁻¹ Pb. Each pot was covered by a 1-2 cm thickness layer of litter (Figure 7.2).



Figure 7.2: Litter placed on the top of the pots.

The seeds were incubated with water in Petri dishes for 24 hours. Then, around 100 seeds were planted in each pot. After two weeks, ten plants were left per pot. Since the goal of the experiment was to focus on the changes in microbial composition in the soil and not on the evaluation on plant growth, during the experiment, seeds were replaced in those treatments where plants died, or a low plant growth occurred (Figure 7.3). This was the case for the S and SB treatments. On the other hand, in those treatments where the plant growth was satisfactory, both SC and SCB, there was not need of plant replacement.

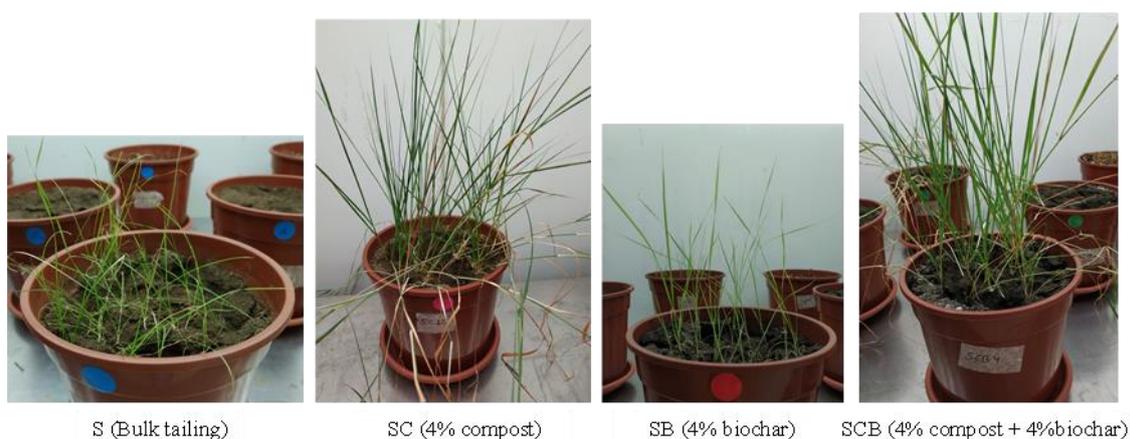


Figure 7.3: Pictures of the pots with plants at the end of the experiment.

In order to avoid any effect on microbial composition related to an excess of humidity, all pots were slightly irrigated by adding 50-80 ml weekly (approximately half of field capacity)

throughout the experiment (12 months) and maintained in a climate chamber with controlled temperature/light/humidity (23°C during 16 h light and 16°C during 8 h darkness; 60 % constant relative humidity). At the end of the experiment, soil samples were taken from each pot. An aliquot of each sample was stored in sterile falcon tubes at -20°C for microbial analysis. The rest was used for the determination of soil parameters. All the plant biomass (roots and shoots) were collected and weighted per each pot.

7.2.2. Soil parameters analyses

For all the samples a 1:2.5 soil (g):water (ml) extraction was performed by shaking for 2 hours. These extracts were filtered through nylon membrane 0.45 µm syringe filters (WICOM) and used for measuring pH, Electrical Conductivity (EC), Dissolved Organic Carbon (DOC) and Dissolved Nitrogen (DN) by using a TOC-automatic analyser (TOC-VCSH Shimadzu), and metals (Cu, Mn, Pb, Zn) by using an ICP-MS (Agilent 7500A).

7.2.3. DNA extraction, PCR amplification and sequencing

Microbial (bacteria and fungi) DNA was extracted from 0.25 g soil using the PowerSoil DNA Isolation Kit (MOBIO), according to the manufacturer's instructions. The isolated DNA was quantified using a NanoDrop 2000 spectrophotometer. Library preparation and Illumina sequencing were carried out at the IPBLN Genomics Facility (CSIC, Granada, Spain). Amplicon libraries targeting the V3-V4 region of the 16S rDNA (bacteria) and ITS2 region (fungi) were generated by a two-steps PCR strategy. Gene-specific amplification was performed in triplicate with 15 ng of soil-extracted DNA in a final volume of 10 µl. Gene specific primers, V3V4fw (5' CCTACGGGNGGCWGCAG 3'), V3V4rev (5' GACTACHVGGGTATCTAATCC 3'), ITS3_KYO2-Fw (5' GATGAAGAACGYAGYRAA 3') and ITS4-Rev (5' TCCTCCGCTTATTGATATGC 3'), were designed with Nextera overhang adapters. Primers were used at a final concentration of 0.2 µM. Reactions were performed with 1X KAPA HiFi Hot Start Ready Mix DNA polymerase (Roche Diagnostics, West Sussex, United Kingdom). Cycling

conditions were 95°C for 3 min; 25 x (95°C for 30 s, 55°C for 30 s, 72°C for 30 s) and then 72°C for 5 min for 16S amplification and 95°C for 3 min; 27 x (95°C for 30 s, 47°C for 30 s, 72°C for 30 s) and then 72°C for 5 min for ITS2 amplification. Triplicates were pooled together and validated through visualization on a 1.8 % (w/v) agarose gel. Amplicons were then purified using NucleoMag® NGS Clean-up and Size Select Kit (Macherey-Nagel, Düren, Germany). A second PCR step attached dual combinatorial indices and Illumina sequencing adapters using Nextera XT v2 index kit. Cycling conditions were 95°C for 3 min, 8 x (95°C for 30 s, 55°C for 30 s, 72°C for 30 s) and then 72°C for 5 min. Amplicon generation was validated again through visualization on a 1.8 % (w/v) agarose gel and cleaned with NucleoMag® NGS Clean-up and Size Select Kit (Macherey-Nagel). Concentration was measured on the Qubit® fluorometer (Thermo). Amplicons were pooled in an equimolecular manner and final library mix was run on a Bioanalyzer HS DNA chip to verify quality and size distribution. The library pool was then diluted and denatured as recommended by Illumina MiSeq library preparation guide. The 300x2nt paired-end sequencing was conducted on a MiSeq sequencer.

7.2.4. Bioinformatics and statistical analyses

Raw sequence data (16S and ITS2) in FASTQ format were subjected to quality control analysis with FastQC software and prepared for taxonomic classification using the Mothur software (version 1.43.0) (Schloss et al., 2009) and following the standard operating protocol proposed by (Kozich et al., 2013). Overlapping pairs of sequence reads were merged into contigs. In addition, reads with ambiguous bases, duplicated contigs and homopolymers longer than 13 bp were removed. The VSEARCH algorithm (embedded in the Mothur framework) was used to remove chimeras and these were subsequently omitted. The resulting sequences were classified according to the taxonomy into the corresponding Operational Taxonomic Units at 97 % similarity, besides using the reference trainset 16_022016.pds from https://mothur.org/wiki/RDP_reference_files for Bacteria and ITS sequences provided by the UNITE ITS database (version 7.2) at <https://unite.ut.ee/repository.php> for Fungi. Undesired

lineages such as Plantae, Animalia, Protista, “unknown” and other were removed. The final sequences were then grouped into taxonomic groups (phylum, order, etc), using the phylotype command in Mothur, which relies upon reference taxonomic outlines to classify sequences to taxonomic bins (Schloss and Westcott, 2011). Relative abundance to different taxonomic levels of each bacterial and fungal group were calculated as percentage from the total count of reads in each sample using the `get.relabund` command in Mothur. Finally, taxa relative abundances for each study site, were calculated by means of the three replicates of each treatment. Phyla and orders (both bacteria and fungi) that showed > 5 % abundance in at least two pot groups were considered.

With the microbial data of abundance at order level, the Shannon-Weaver index (H') was calculated (Shannon and Weaver, 1963) as it follows:

$$H'p = - \sum_{i=1}^S p_i \ln p_i$$

Where p_i is the relative frequency of the order “i” at each plot and S is the number of bacteria or fungi orders at each sample.

Statistical analyses were performed with the software IBM SPSS Statistics 24. Homogeneity of variances was tested using Levene's test and data were transformed as needed to fit to a normal distribution. ANOVA with Tukey's test was used in order to evaluate differences among treatments. Data on bacterial and fungal orders abundances were analysed by Canonical Correspondence Analysis (CCA) for evaluating the relationship between selected soil parameters and microorganisms. The CANOCO software for Windows v4.02 was used for CCA (Ter Braak and Smilauer, 1999).

7.3. Results and discussion

The initial soil conditions, including microbial composition, for this current experiment corresponded to the data shown at the final stage of the previous Chapter 6. The starting conditions for the new treatments are shown in the Table 7.1 for soil parameters and in the Figures 7.4 and 7.5 for the bacterial and fungal composition, respectively.

The treatments containing compost, both SC and SCB, showed higher EC, DOC and DN values than the other two treatments ($p < 0.05$). The treatment with biochar-only, SB, showed lower concentrations ($p < 0.05$) of water extractable Mn and Zn compared to the bulk tailings treatment, S.

Table 7.1: Parameters measured in the 1:2.5 soil:water extract for the four treatments (S, bulk tailings; SC, tailings + 4 % compost; SB, tailings + 4 % biochar; SCB, tailings + 4 % biochar + 4 % compost). EC is Electrical conductivity; DOC is Dissolved Organic Carbon; DN is Dissolved Nitrogen. Data are average \pm standard error of three repetitions. Different letters among columns in the same row indicate significant differences (ANOVA, Tukey's test, $p < 0.05$).

SOIL PARAMETERS	TREATMENT				
	S	SC	SB	SCB	
pH	7.53 \pm 0.05	7.41 \pm 0.04	7.59 \pm 0.05	7.56 \pm 0.02	
EC (dS m ⁻¹)	2.10 \pm 0.03 a	2.50 \pm 0.08 b	2.15 \pm 0.04 a	2.48 \pm 0.03 b	
DOC (mg kg ⁻¹)	6.4 \pm 0.7 a	23.9 \pm 0.8 b	7.8 \pm 2.0 a	26.4 \pm 1.9 b	
DN (mg kg ⁻¹)	5.0 \pm <0.01 a	64.5 \pm 3.2 c	5.0 \pm <0.01 a	23.9 \pm 3.2 b	
Water extractable metal(loid) concentrations (μ g kg ⁻¹)	Mn	39.1 \pm 5.5 b	22.0 \pm 6.3 ab	17.3 \pm 4.3 a	12.4 \pm 0.3 a
	Cu	20.1 \pm 2.1 a	26.6 \pm 1.6 ab	18.9 \pm 1.2 a	30.3 \pm 2.9 b
	Zn	206.8 \pm 4.8 b	194.5 \pm 10.9 a	121.6 \pm 11.1 b	103.7 \pm 6.4 a

In relation to the bacterial composition (Figure 7.4), the most abundant phyla corresponded to *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Planctomycetes*, *Bacteroidetes* and *Gemmatimonadetes*. While the *Proteobacteria*, *Planctomycetes* and *Bacteroidetes* phyla showed higher relative abundances in those pots containing compost (SC and SCB), the *Actinobacteria* and *Gemmatimonadetes* phyla were more abundant in those pots without compost (S and SB, $p < 0.05$). The *Acidobacteria* phylum showed higher relative abundance percentages in the non-amended pots (S) than in those pots containing compost (SC and SCB) ($p < 0.05$, Figure 7.4 and Chapter 6).

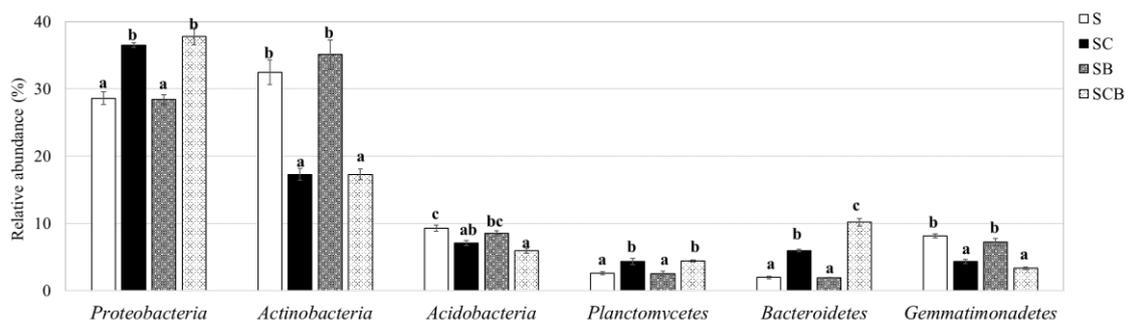


Figure 7.4: Bacterial composition for the four treatments (S, bulk tailings; SC, tailings + 4 % compost; SB, tailings + 4 % biochar; SCB, tailings + 4 % biochar + 4 % compost). Bars on columns indicate standard error (n=3). Different letters among columns indicate significant differences (ANOVA, Tukey's test, $p < 0.05$).

In the case of fungal composition, *Ascomycota* and *Basidiomycota* were the most abundant phyla (Figure 7.5). Both fungal groups showed lower relative abundance percentages ($p < 0.05$) in those pots only amended with compost (SC) than in those without compost addition (S and SB).

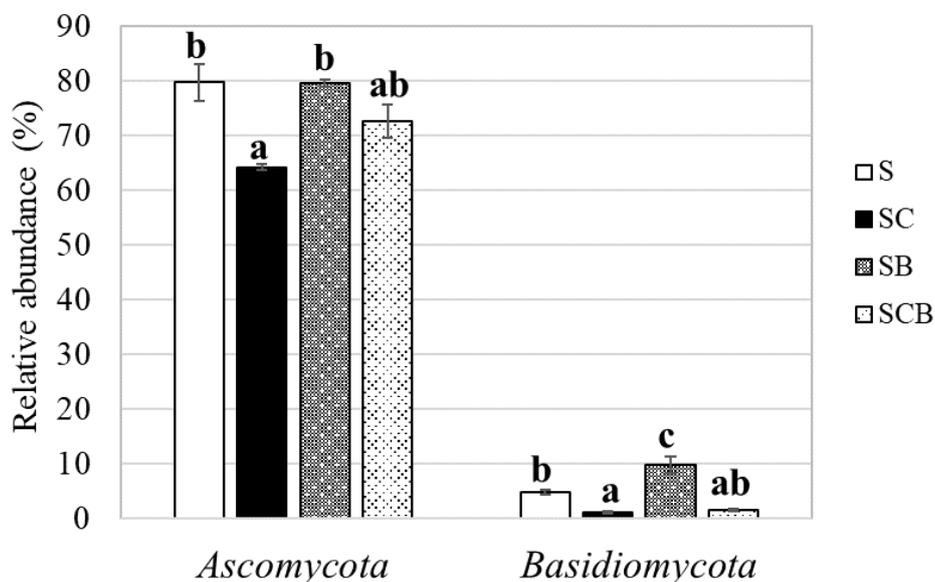


Figure 7.5: Fungal composition for the four treatments (S, bulk tailings; SC, tailings + 4 % compost; SB, tailings + 4 % biochar; SCB, tailings + 4 % biochar + 4 % compost). Bars on columns indicate standard error (n=3). Different letters among columns indicate significant differences (ANOVA, Tukey's test, $p < 0.05$).

The effects on soil parameters and microbial composition of the biochar/compost amendments obtained during the six months of incubation were already reported and discussed

in the Chapter 6. Those results showed that the addition of compost caused stronger effects in most of soil parameters and microbial composition than the biochar, especially at the initial stage of the experiment (1-3 months). However, the higher dependence on labile organic carbon for some bacterial groups at the treatments containing compost determined their shifts along time and their substitution by other taxa reliable of recalcitrant organic matter. Unlike bacteria, only a few fungal orders increased their relative abundances in the treatments containing compost, while the rest showed a decrease or no clear effect. Taking into account these previous results, this new chapter focuses on the effect of the presence of plant litter (L) and plant growth (P) on selected soil parameters and on microbial composition for each treatment, S, SC, SB and SCB.

7.3.1. Effects of litter and *P. miliaceum* on edaphic parameters

The results of some selected soil parameters are shown in the Table 7.2. The pH values were in the range of neutral-slightly alkaline soils (7.4-7.6). The addition of litter did not cause any significant effect on the pH values ($p > 0.05$) for any amended/non-amended treatment. However, planted pots showed higher pH values than bare or litter contained pots in the bulk tailing (S-P) and single compost (SC-P) treatments. It is known that plant roots may modulate soil pH by balancing ions within the rhizosphere by ion exchange, release of organic exudates and/or respiration (Hinsinger et al., 2003; Li et al., 2016). This effect on pH was not observed in both biochar contained treatments, SB-P and SCB-P, probably because of the higher buffer capacity of the biochar (Fellet et al., 2011; Rinklebe et al., 2016). This buffer effect was absent in the bulk tailings pots, which could have facilitate, in spite of the low plant growth (Figure 7.6), the “detection” of the slight change of pH promoted by plants. The planted pots did not show any effect on electrical conductivity values in any amended/non-amended treatment. However, the compost-only containing treatment with litter (SC-L) showed higher values ($p < 0.05$) than the corresponding planted (SC-P) and bare pots (SC-A).

Table 7.2: Results of the edaphic parameters analysed in the 1:2.5 soil water extract in each treatment (bare, with plant or with litter) from each soil (S, SC, SB and SCB): pH, Electrical Conductivity (EC), Dissolved Nitrogen (DN) and Organic Carbon (DOC) and some metals (Mn and Zn) concentration. Data are average \pm standard error (n=3). Different letters among columns in the same row indicate significant differences (ANOVA, Tukey's test, $p < 0.05$).

TREATMENTS	SOIL PARAMETERS						Water extractable metal concentrations ($\mu\text{g kg}^{-1}$)	
	pH	EC (dS m^{-1})	DOC (mg kg^{-1})	DN (mg kg^{-1})				
					Mn	Zn		
S-A	7.47 \pm 0.01 a	2.28 \pm 0.01	14.4 \pm 1.9 a	6.1 \pm 1.1	13.2 \pm 1.5 a	232.8 \pm 14.0		
S-L	7.46 \pm 0.01 a	2.29 \pm 0.01	25.3 \pm 2.0 b	5.8 \pm 0.6	15.0 \pm 3.1 ab	260.3 \pm 10.1		
S-P	7.60 \pm <0.01 b	2.28 \pm 0.01	11.3 \pm 1.0 a	6.8 \pm 0.7	22.7 \pm 1.3 b	267.5 \pm 5.3		
SC-A	7.49 \pm 0.01 a	2.46 \pm 0.02 a	17.8 \pm 1.6 a	32.3 \pm 1.4 b	8.9 \pm 2.5	249.3 \pm 14.2		
SC-L	7.45 \pm 0.02 a	2.55 \pm 0.02 b	23.0 \pm 1.8 ab	32.3 \pm 0.9 b	8.4 \pm 1.8	285.3 \pm 7.1		
SC-P	7.63 \pm 0.02 b	2.40 \pm 0.02 a	28.0 \pm 1.3 b	7.5 \pm 0.2 a	11.8 \pm 1.1	268.1 \pm 1.0		
SB-A	7.57 \pm 0.01	2.27 \pm 0.02	14.0 \pm 1.9	6.2 \pm 1.2	16.3 \pm 4.4	182.9 \pm 9.0		
SB-L	7.55 \pm 0.02	2.27 \pm 0.01	16.0 \pm 0.6	5.3 \pm 0.2	11.5 \pm 2.3	182.7 \pm 4.1		
SB-P	7.60 \pm 0.02	2.26 \pm 0.01	11.4 \pm 0.4	5.0 \pm <0.1	12.2 \pm 0.4	172.4 \pm 7.8		
SCB-A	7.53 \pm 0.02	2.56 \pm 0.05	25.4 \pm 1.4	15.7 \pm 2.2 b	8.7 \pm 1.2	198.5 \pm 13.6		
SCB-L	7.54 \pm <0.01	2.50 \pm 0.06	25.0 \pm 1.4	10.7 \pm 1.3 ab	8.4 \pm 0.7	223.5 \pm 4.8		
SCB-P	7.54 \pm 0.01	2.33 \pm 0.02	23.4 \pm 2.0	7.5 \pm 0.6 a	11.1 \pm 0.9	192.0 \pm 8.9		

The treatments containing biochar (SB and SCB) did not show any significant effect on DOC with regards to the presence of litter or plants. In the bulk tailings treatment (S), the presence of litter (S-L) significantly increased DOC concentrations ($p < 0.05$) in relation to the bare (S-A) and planted pots (S-P). This increase could be attributed to the mineralization of the most labile fraction of the litter (Purahong et al., 2016; Herzog et al., 2019) and the low background DOC values in these non-amended treatments. The compost-only containing treatments (SC) showed higher DOC concentrations ($p < 0.05$) in the planted pots (SC-P) than the bare pots (SC). This could be explained by the contribution of plant root exudates (Haichar et al., 2016; Colin et al., 2019). In this case, the contribution of the labile fraction of the litter (SC-L) was not enough to provoke differences in relation to the bare pots (SC-A) probably because of the higher background DOC contents of the SC treatments.

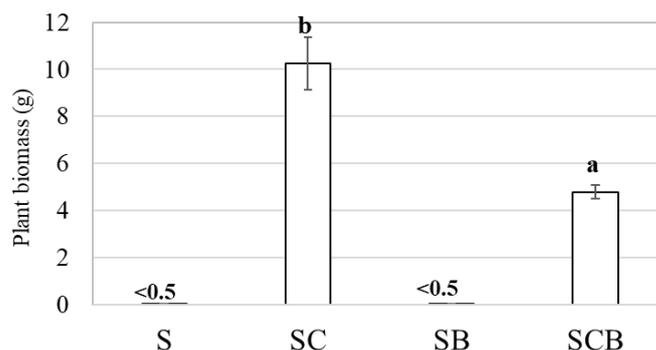


Figure 7.6: Plant biomass recorded in the four treatments planted pots (S-P, SC-P, SB-P and SCB-P). Bars on columns indicate standard errors (n=3). Different letters among columns indicate significant differences (ANOVA, Tukey's test, $p < 0.05$).

No effect of litter or plants was detected for DN in the bulk (S) and single biochar (SB) treatments. However, in the treatments containing compost (both, SC and SCB) some differences occurred: in the SC treatment, the pots with plants (SC-P) showed 4-fold lower DN concentrations than the litter (SC-L) and bare (SC-A) pots; in the SCB treatment, the planted pots showed about half ($p < 0.05$) of the DN concentrations of the bare pots (SC-A), being the litter pots intermediate between them ($p > 0.05$, Table 7.2). This lower DN concentration (mostly attributed to NO_3^-) in the planted pots, could be explained by a more effective N absorption by plants. *Piptatherum miliaceum* has been described as an efficient plant in uptaking available soil nitrogen and fast response in producing biomass in amended soils (Arco-Lázaro et al., 2017; Martínez-Oró et al., 2019).

Lead and copper water extractable concentrations were below the detection limit for all the samples ($< 10 \mu\text{g kg}^{-1}$). No effect of litter or plants was detected for Zn in all the treatments. For Mn, only in the non-amended treatment (S), the planted pots (S-P) showed higher Mn concentrations ($p < 0.05$) than the bare pots (S-A, Table 7.2).

7.3.2. Effects of litter and *P. miliaceum* on bacterial composition

The effects of the amendments (biochar/compost) on the microbial composition were already discussed in the previous Chapter 6. In this section, comparative effects of litter/plants in relation to bare soil pots are shown and discussed.

The relative abundance of two bacterial phyla, *Proteobacteria* and *Actinobacteria*, accounted for more than 50 % of total bacterial abundance (Figure 7.7). Taxa belonging to these two phyla are known to be predominant in metal enriched environments (Narendrula-Kotha and Nkongolo, 2017; Rosenfeld et al., 2018; Sun et al., 2018).

The *Proteobacteria* phylum showed relative abundances of around 30 % for all the amended/non-amended treatments (Figure 7.7). In those treatments containing compost (both SC and SCB) the planted pots (SC-P and SCB-P) showed lower percentages of *Proteobacteria* relative abundance ($p < 0.05$) than the corresponding bare and litter containing pots. This was due to the higher abundance ($p < 0.05$) of *β -proteobacteria uncl.* and *γ -proteobacteria uncl.* orders at the bare pots (Figure 7.8, 4 % and 8 % relative abundance, respectively) compare with the planted pots (2 % relative abundance for both orders). These two bacterial orders, especially *β -proteobacteria uncl.*, are considered copiotrophic (Fierer et al., 2007) and have been previously identified in unvegetated mine tailings at the Cartagena-la Unión Mining District (Risueño et al., 2020, Chapter 4). Contrary to what occurred for the *β -proteobacteria uncl.* and *γ -proteobacteria uncl.* orders, the *Rhizobiales* and *Sphingomonadales* orders, which also belong to the *Proteobacteria* phylum, showed higher relative abundance percentages in the planted pots (SC-P and SCB-P) than in the unplanted pots. These two orders are associated to plant rhizospheres and show a strong dependence on root exudates (Bulgarelli et al., 2013; Haichar et al., 2016). Therefore, the higher growth of plants in the compost containing treatments (Figure 7.6) might explain the higher occurrence of these two orders and the depletion, probably by competition, of the copiotrophic *β -proteobacteria uncl.* and *γ -proteobacteria uncl.* orders. By contrast, in those treatments without compost (S and SB), in which there was a scarce development of plants,

significant differences were not detected between the *Rhizobiales* and *Sphingomonadales* relative abundances in planted and unplanted pots. Interestingly, those non-amended and biochar-only containing pots with litter (S-L and SB-L, respectively) showed higher relative abundances of the copiotrophic orders, *β -proteobacteria uncl.* and *γ -proteobacteria uncl.* (Figure 7.8) than the bare (S, SB) and planted (S-P, SB-P) pots. In this case, we may hypothesize that the litter acted as a carbon source (Table 7.2, higher DOC in S-L pots), which has stimulated the growth of those well-known copiotrophic orders.

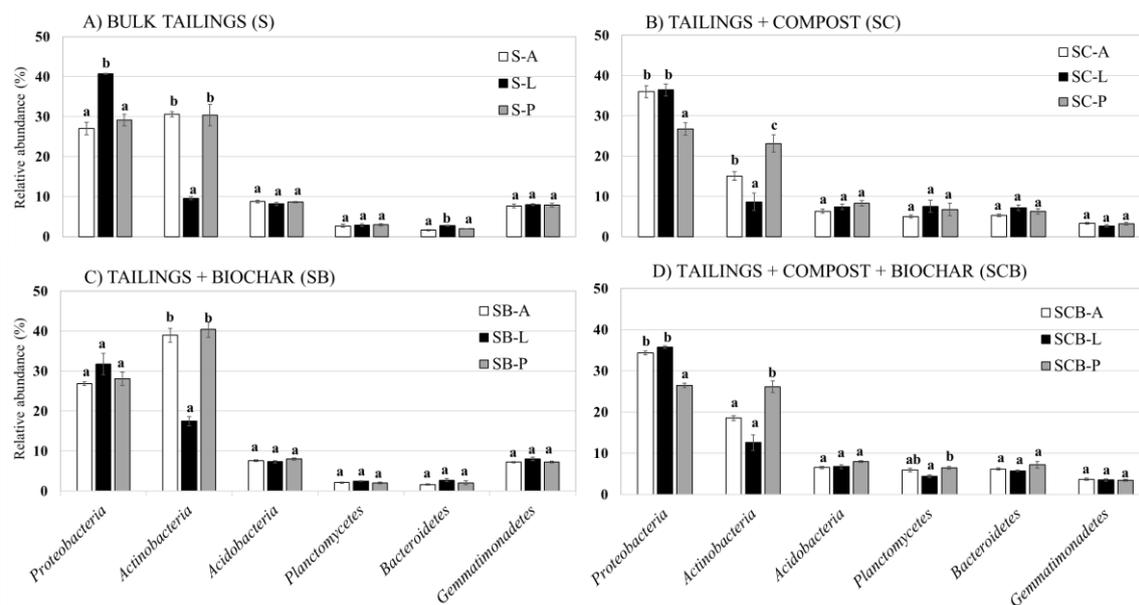


Figure 7.7: Relative abundance percentages of bacterial phyla in each treatment (bare, with plants or with litter) at each kind of soil (S, SC, SB and SCB; figure A, B, C or D, respectively). Bars on columns indicate standard error (n=3). Different letters among columns indicate significant differences (ANOVA with Tukey's test, $p < 0.05$).

In relation to the *Actinobacteria* phylum, those treatments without compost, both S and SB, which contained litter (S-L and SB-L) showed lower relative abundance percentages ($p < 0.05$, Figure 7.7) than the bare (S-A and SB-A) and planted pots (S-P and SB-P). The three identified orders which belong to this phylum, *Actinobacteria uncl.*, *Acidimicrobiales* and *Actinomycetales*, showed that performance in the non-amended pots (S). This fact was also true for *Actinobacteria uncl.* and *Actinomycetales* at the biochar-only containing pots (SB). This decrease in the relative abundances promoted by the litter could be explained by the oligotrophic character of these

orders, whose abundance is favoured in extreme environments but negatively affected in systems with availability of organic matter (in our case, the presence of litter), where fast-growing bacteria may show a better competitive behaviour (Burgess et al., 2020) (in the previous Chapter 6, these bacterial orders were negatively affected in those treatments containing compost compared to the bulk tailings). Unlike S and SB treatments, the treatments with compost, both SC and SCB, showed higher relative abundances of the *Actinobacteria* phylum ($p < 0.05$) in the planted pots (SC-P, SCB-P) compared with the unplanted pots (SC-A, SC-L, SCB-A-SCB-L). This was mainly due to the contribution of the *Actinomycetales* order (Figure 7.8) and it could be explained because of the higher plant growth observed in the treatments containing compost (Figure 7.6) and the strong relation of this bacterial order with plant rhizospheres (Santoyo et al., 2016; Bhatti et al., 2017; Hozzein et al., 2019). At this point, it is interesting to note that, as it was shown in the Chapter 6, the *Actinomycetales* order was one of the groups more negatively affected by the addition of compost. In the current experiment, this order was the one which showed the higher increase in relative abundances in relation to the bare or litter pots, suggesting a key role in the development of the *P. miliaceum* plants. This revealed that the microbiome promoted by the addition of composts could not fit with those specific bacterial groups which belong to the plant rhizospheres. However, as it was shown in our experiment, plants showed a strong ability to shape the microbiome of rhizospheres enhancing specific bacterial groups (Guo et al., 2019; Zeng et al., 2019) and depleting other bacterial lithotrophic groups (Li et al., 2016; Valentín-Vargas et al., 2018).

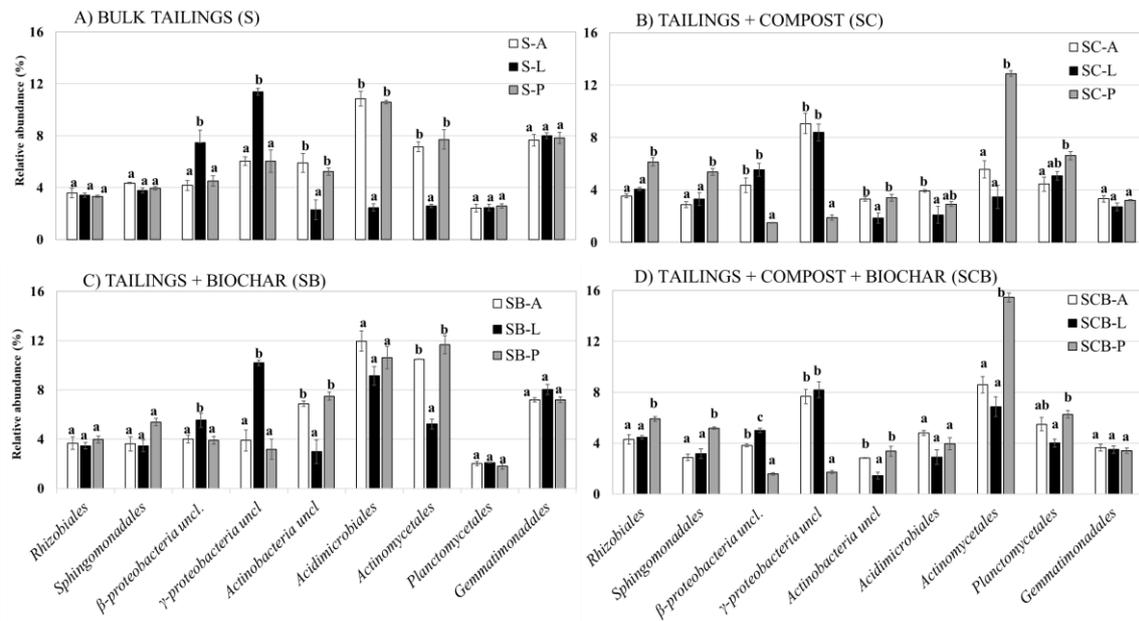


Figure 7.8: Relative abundance percentages of bacterial orders in each treatment (bare, with plants or with litter) at each kind of soil (S, SC, SB and SCB; figure A, B, C or D, respectively). Bars on columns indicate standard error ($n=3$). Different letters among columns indicate significant differences (ANOVA with Tukey's test, $p < 0.05$).

The *Acidobacteria*, *Planctomycetes*, *Bacteroidetes* and *Gemmatimonadetes* phyla showed relative abundances lower than 10 % for all the treatments (Figure 7.7). In the case of the *Acidobacteria* and *Gemmatimonadetes* phyla (including the *Gemmatimonadales* order) effect of litter or plant was not observed ($p > 0.05$). The relative abundances of the *Bacteroidetes* phylum were not affected by litter or plant presence in the amended treatments (biochar and/or compost, $p < 0.05$). However, the bulk tailings treatment pots with litter showed higher relative abundances values ($p < 0.05$) than the bare and planted pots. This could be explained because the contribution of the litter to the total organic content of the soil would result more relevant in the non-amended pots than in the amended pots. In addition, this could have favoured the activity of taxa belonging to the *Bacteroidetes* phylum, which are known to act as fast-acting decomposers, especially during the initial stages of organic matter decomposition (Naumoff and Dedysh, 2012; Herzog et al., 2019). The relative abundances of the *Planctomycetes* phylum (including the *Planctomycetales* order) did not show any difference ($p > 0.05$) between the presence of litter or plants in the non-amended (S) and biochar-only treatments (SB). However, some differences were detected among the treatments containing compost (SC and SCB), whose relative abundances

were already about 2-fold higher than in the non-amended (S) and biochar-only (SB) treatments. The taxa belonging to this phylum can act as nutrient recyclers (Nie et al., 2015; Oka and Uchida, 2018) and are known to proliferate in soils with available supply of organic carbon (Meglouli et al., 2018). This might explain its higher relative abundances in the two treatments containing compost.

For a better understanding of the comparison among the different treatments a CCA was performed (Figure 7.9, Tables 7.3 and 7.4). The CCA was significant (Monte-Carlo test, $p < 0.05$) and the first axis explained 61.3 % of the variance.

Table 7.3: Data of the Canonical Correspondence Analysis (CCA) including eigenvalues of the two first axis and cumulative percentage of variance of bacteria-soil data interaction at all treatments. The significance of the first canonical axis (Monte-Carlo test, p -value) is also provided.

Parameter evaluated	Axis	Eigenvalue	% cumulative variance	Significance
Bacterial orders	I	0.069	61.3	0.002
	II	0.036	93.9	

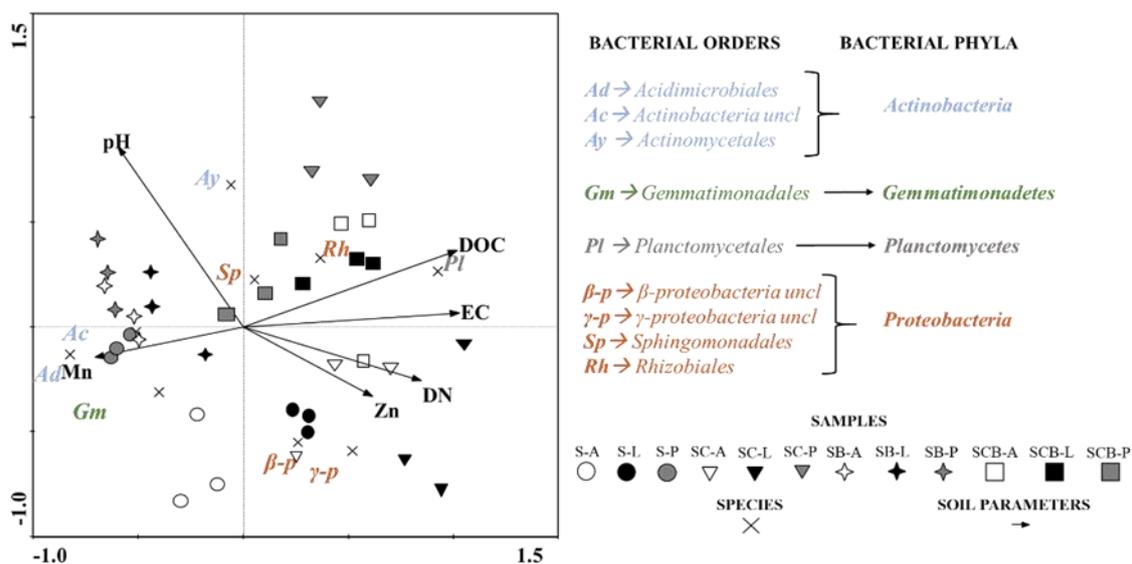


Figure 7.9: Ordination diagram obtained for the Canonical Correspondence Analysis (CCA) of bacterial orders abundance and soil properties from all treatments. Mn and Zn represent water extractable concentrations; “DOC” is Dissolved Organic Carbon concentration; “DN” is Dissolved Nitrogen concentration; “EC” and “pH” are Electrical Conductivity and pH, respectively. All soil parameters were analysed in the 1:2.5 soil:water extract ($n=3$).

The positive side of the CCA1-axis was defined by higher EC values, and increasing concentrations of DOC and DN ($r > 0.63$ for EC, DOC and DN) and to a lesser extent the water extractable Zn concentrations ($r = 0.46$, Table 7.4). This result may indicate that those parameters, which defined better fertile soil conditions, favour the long-term increase of *Planctomycetes* taxa, perhaps due to its implication on recycled of nutrients (Nie et al., 2015; Herzog et al., 2019). In contrast, the negative side of the CCA1-axis was conditioned by lower values of the aforementioned parameters and higher pH values and water extractable Mn concentrations ($r = -0.44$ and $r = -0.53$, respectively) (Table 7.4). This situation represents less favourable soil fertility conditions and seems to have favoured those oligotrophic bacterial orders, such as *Acidimicrobiales* (Sun et al., 2019) or *Gemmatimonadales* (Burgess et al., 2020) (Figure 7.9). This way, the CCA1-axis (Figure 7.9) segregated the bacterial orders in two groups: one group more abundant in the treatments without compost (circles, S, and stars, SB) and another one with higher relative abundances in the compost containing treatments (inverted triangles, SC, and squares, SCB). The positive side of the CCA2-axis was mainly defined by higher pH values ($r = 0.52$). This axis segregated the samples from bare and litter pots coming from S and SC soils (negative side), from those where plants were included (S-P and SC-P, Figure 7.9).

Table 7.4: Weighted correlation matrix for the first two species axes and environmental variables for bacterial orders.

Soil parameters	Bacterial orders	
	Species Ax1	Species Ax2
pH	-0.44	0.52
Electrical Conductivity (EC)	0.77	0.04
Dissolved Organic Carbon (DOC)	0.76	0.22
Dissolved Nitrogen (DN)	0.63	-0.16
Water extractable metals	Mn	-0.53
	Zn	0.46

The CCA Figure (Figure 7.9) revealed that the highest effect of plants in the bacterial composition occurred in those treatments containing compost (inverted triangles, SC, and squares, SBC). These samples are represented by the grey inverted triangles and grey squares. While white (bare) and black (litter) inverted triangles appeared together in the graph, the grey inverted triangles (plants) were separated indicating a different bacterial composition. This fact also occurred for the grey squares (plants) in relation to the white (bare) and black (litter) squares. Unlike plants, the highest effect of litter occurred in the non-amended treatment (circles, S): along the CCA2-axis the black circles (litter) were depicted separately from white (bare) and grey (plants) circles.

7.3.3. Effects of litter and *P. miliaceum* on fungal composition

As it was shown in the previous Chapter 6, the fungal composition was mainly formed by taxa belonging to the *Ascomycota* and *Basidiomycota* phyla (Figure 7.10). These two phyla are known to include fungal groups with special relevance in metal(loid) polluted soils (Op De Beeck et al., 2015; Narendrula-Kotha and Nkongolo, 2017; Rosenfeld et al., 2018).

The *Ascomycota* phylum showed relative abundance percentages higher than 60 % at all amended/non-amended treatments (except in the bare pots of the SB treatment, SB-A, which accounted around 40 %). The presence of litter or plants provoked no significant effects ($p > 0.05$) at the phylum level for the non-amended (S) and the compost containing treatments (SC and SCB). The bare pots (SB-A) showed lower relative abundances than the litter (SB-L) and planted pots (SB-P) in the biochar-only containing treatment (SB).

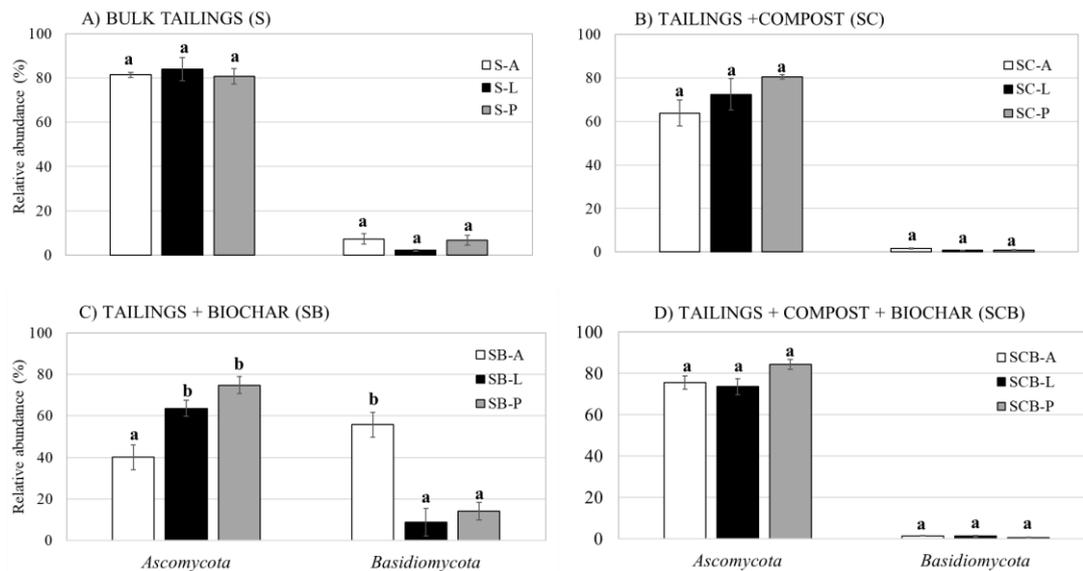


Figure 7.10: Relative abundance percentages of fungal phyla from each treatment (bare, with plants or with litter) at each kind of soil (S, SC, SB and SCB; figure A, B, C or D, respectively). Bars on columns indicate standard error (n=3). Different letters among columns indicate significant differences (ANOVA with Tukey's test, p<0.05).

Although the fungal order diversity of the single compost containing treatment was the only one that showed significant differences (the SC-P diversity was higher than those at SC-A and SC-L pots, p<0.05), for all the non-amended and amended treatments, some significant differences took place when the relative abundance at order level was evaluated. Eight fungal orders belonging to *Ascomycota*, which are related to both saprophytic and endophytic behaviour (Webster and Weber, 2007), were detected: *Hypocreales*, *Sordariales*, *Sordariomycetes uncl.*, *Microascales*, *Eurotiales*, *Chaetothyriales*, *Ascomycota uncl.* and *Pleosporales* (Figure 7.11). In the non-amended (S) and biochar-only (SB) containing treatments, the litter (S-L and SB-L) and planted (S-P and SB-P) pots showed higher relative abundances of the *Hypocreales* order (up to 3-fold higher, p<0.05) than the bare (S-A and SB-A) pots. Similar results were observed for the *Sordariales* order between the bare and litter pots in the non-amended (S) and compost (SC and SCB) containing treatments. These increases in the litter pots might be explained by the specific ability of these two orders to proliferate in the presence of organic matter (higher DOC concentrations in these pots, Table 7.2). Both fungal orders, *Hypocreales* and *Sordariales*, are considered as plant material decomposers: while those taxa belonging to the *Sordariales* order are known to be more dependent of labile organic matter and more relevant in the early stages of

decomposition, those of the *Hypocreales* order are more specialised in degrading more complex lignocellulose materials during late stages of decomposition (Ma et al., 2013). In the SB treatment no significant effects of litter and plants were detected ($p > 0.05$) for the rest of the *Ascomycota* orders (Figure 7.11). In the non-amended treatment, S, the presence of litter (S-L) decreased by half the relative abundance percentages of *Chaetothyriales* and *Pleosporales* in relation to the bare pots (S-A, $p < 0.05$). The higher DOC values at the litter pots could have displaced these orders, favouring other fungal taxa with better competitive behaviour (e.g. *Hypocreales* or *Sordariales*). This revealed the strong relationship between fungi and the C source, which could have modulated the interaction among fungal groups and shaped their composition (Bastida et al., 2013, 2015). However, the higher plant growth recorded in the treatments containing compost, both SC and SCB (Figure 7.6), favoured a higher relative abundance of those two orders (*Chaetothyriales* and *Pleosporales*) in the planted pots (SC-P and SCB-P) compared with the bare (SC-A and SCB-A) and litter (SC-L and SCB-L) pots (Figure 7.11). This could be explained by the wide range of lifestyles (saprophytes, plant endophytes) of the taxa included in the *Pleosporales* (Webster and Weber, 2007) and *Chaetothyriales* (Chen et al., 2015; Teixeira et al., 2017) orders. In fact, taxa belonging to the *Pleosporales* order has been described to promote plant growth in saline environments (Murphy et al., 2015) and is known their behaviour as endophytes for most of plants (Kolaříková et al., 2017).

The relative abundances of the *Basidiomycota* phylum were lower than 10 % in all the treatments (S, SC, SCB) except in the biochar-only containing treatment (SB). In this treatment, the bare pots (SB-A) showed higher relative abundance percentages ($p < 0.05$) than the litter (SB-L) and planted (SB-P) pots (Figure 7.10). This higher abundance was mainly due to the contribution of the *Agaricales* order (Figure 7.11). The saprophytic fungal taxa of the *Basidiomycota* phylum are involved on hard to decompose organic materials, which normally cannot be accessed by taxa of the *Ascomycota* phylum (Lundell et al., 2010). This could explain that in the presence of litter or plants (even if there is no significant effects in DOC values) the

taxa belonging to Basidiomycota might be displaced by others of Ascomycota (Osono and Takeda, 2006; Herzog et al., 2019).

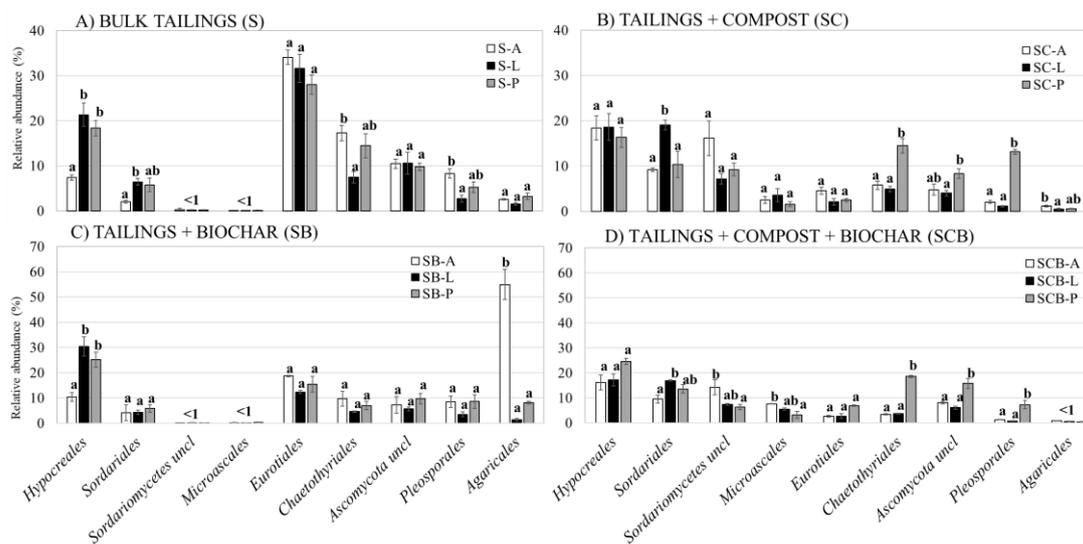


Figure 7.11: Relative abundance percentages of fungal orders from each treatment (bare, with plants or with litter) at each kind of soil (S, SC, SB and SCB; figure A, B, C or D, respectively). Bars on columns indicate standard error (n=3). Different letters among columns indicate significant differences (ANOVA with Tukey's test, $p < 0.05$).

For a better understanding of the comparison among the different treatments a CCA was done (Figure 7.12 and Table 7.5 and 7.6). The CCA was significant (Monte-Carlo test, $p < 0.05$) and the first axis explained 68.4 % of the variance.

Table 7.5: Data of the Canonical Correspondence Analysis (CCA) including eigenvalues of the two first axis and cumulative percentage of variance of fungal-soil data interaction at all treatments. The significance of the first canonical axis (Monte-Carlo test, p-value) is also provided.

Parameter evaluated	Axis	Eigenvalue	% cumulative variance	Significance
Fungal orders	I	0.283	68.4	0.002
	II	0.91	90.4	

Table 7.6: Weighted correlation matrix for the first two species axes and environmental variables for fungal orders.

Soil parameters	Fungal orders	
	Species Ax1	Species Ax2
pH	-0.17	-0.28
Electrical Conductivity (EC)	0.84	0.01
Dissolved Organic Carbon (DOC)	0.63	0.13
Dissolved Nitrogen (DN)	0.66	-0.03
Water extractable metals	Mn	-0.63
	Zn	0.23

7.4. Conclusions

Our experiment showed that the effects of plant and litter in microbial composition were dependent on the previous presence of amendments, especially compost. At those treatments containing compost, the higher growth of plants was able to shape microbial composition (both, bacterial and fungi), while at the non-amended and biochar-only containing treatments (where a scarce growth of plants occurred), litter showed a more important role, especially in shaping bacterial composition. The higher growth of plants favoured some bacterial groups, whose relative abundances were depleted in the compost containing treatments (e.g. *Actinomycetales*) and other groups related to plant rhizospheres (e.g. *Rhizobiales*). This result revealed the ability of plants for shaping their own rhizospheric microbiome when the amendments contribute to ameliorate the low fertility of mine tailing soils. The employment of the combinations biochar-compost could result optimal to achieve the establishment of a vegetation cover at tailings, providing at the same time an effective tool to stimulate plant growth (higher plant biomass), a recalcitrant source of organic matter (biochar) and a support for the development of a plant rhizospheric microbiome.

7.5. Acknowledgements

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CHAPTER 8. Final conclusions

Based on the objectives proposed in Chapter 3 and the results obtained and described in Chapters 4, 5, 6 and 7, the general conclusions of this PhD Thesis are the following:

- I. In relation to the factors which determine soil functionality in the phytomangement of semiarid mine tailing piles, it can be concluded that the presence of spontaneous vegetation provoked a positive effect on soil fertility and microbiological indicators, stimulating the presence of bacterial orders involved in biogeochemical cycles. The resulting edaphic functionality in these impacted sites may support its long-term sustainability.
- II. In relation to the identification of the edaphic factors which condition the establishment of spontaneous vegetation at mine tailings, it can be concluded that the heterogeneous edaphic conditions, including the microbiological, lead plant species to develop specific strategies for colonization. In the specific case of neutral pH tailings, salinity seemed to play a more determining role than metal concentrations in the establishment of vegetation. As a consequence, the establishment of early ruderal colonizers such as *Zygophyllum fabago* could be limited to high salinity areas with most adverse soil conditions for plant growth, while late successional plant species such as *Pinus halepensis* or *Tetraclinis articulata* may selectively colonize more favourable tailings patches. Specific halotolerant bacterial and fungal taxa within plant rhizospheres might result a key factor to facilitate the establishment of vegetation at neutral pH tailings. In terms of phytomangement, this selective colonization of the different plant species in tailings would allow increasing their biodiversity and thus their resilience against environmental stressors.
- III. In relation to the effects of soil organic amendments on the microbial composition (bacteria and fungi) of mine tailings, it can be concluded that the type of C source (labile/recalcitrant) provided by the amendment and the changes of labile organic matter concentrations along with the time played a critical role in determining the microbial composition. The combination of amendments with high contents of easily degradable

organic matter, such as compost, and high recalcitrant carbon concentrations, such as biochar, could result the best option to improve the long-term soil functionality at mine tailings. While compost may provoke an increase of bacterial diversity and therefore favour the resilience of the system against environmental stressors, biochar could generate a storage of carbon through the introduction of large amounts of recalcitrant organic matter. These conditions may allow the further development of some microbial groups implicated in the degradation of complex organic substrates, which also play an important role on plant establishment and vegetation development.

- IV. In relation to the effects of the presence of plants and litter on amended/non-amended tailings on microbial composition (bacteria and fungi), it can be concluded that in tailings amended with amendments type compost the higher growth of plants was able to shape microbial composition (both, bacteria and fungi). This revealed the ability of plants for shaping their own rhizospheric microbiome when amendments contribute to ameliorate the low fertility of mine tailing soils. Under a scarce growth of plants which occurred at non-amended conditions or with amendments type biochar, the presence of litter showed a more important role, especially in shaping bacterial composition. The employment of the combinations biochar-compost could result optimal to achieve the establishment of a vegetation cover at tailings, providing at the same time an effective stimulation on plant growth and a recalcitrant source of organic matter without negatively affecting the specific plant microbiomes.

Finally, future research could be focused on pilot-scale trials which include the application of amendments and the response of specific plant species in the real climate conditions of the tailings area.