RESTAURACIÓN DE SUELOS CONTAMINADOS POR ELEMENTOS TRAZA: EFECTO DE LA VEGETACIÓN ARBÓREA EN LAS COMUNIDADES DE HONGOS DEL SUELO

Memoria presentada por

MARTA GIL MARTÍNEZ

para optar al grado de Doctora por la

Universidad de Sevilla

Facultad de Química

Departamento de Cristalografía, Mineralogía y Química Agrícola

Programa de Doctorado de Recursos Naturales y Medio Ambiente

Directores

Teodoro Marañón Arana

María Teresa Domínguez Núñez

Sevilla, 8 de octubre de 2020

Este trabajo de investigación ha sido financiado por:

Beca para contrato predoctoral (FPI) dentro del Programa Estatal de Promoción del Talento y su Empleabilidad en I+D+i, en el marco del Plan Estatal de Investigación Científica y Técnica y de Innovación 2013-2016. Referencia: BES-2015-073882. Financiado por el Ministerio de Ciencia, Innovación y Universidades y el Fondo Social Europeo.

Proyecto RestEco – Restauración y provisión de servicios ecosistémicos en suelos degradados en el Corredor Verde del Guadiamar (Sevilla, España). Proyecto Retos. Referencia: CGL2014 – 52858 - R. Financiado por el Ministerio de Ciencia, Innovación y Universidades.

Proyecto RECARE – Evaluación de las amenazas actuales de los suelos y búsqueda de soluciones para prevenir la degradación de los suelos en Europa. Caso de estudio: Contaminación del suelo por metales pesados en el Corredor Verde del Guadiamar (Sevilla, España). ENV.2013.6.2-4 'Sustainable land care in Europe'. Referencia: 603498. Financiado por el 7º Programa Marco de Investigación, Desarrollo Tecnológico e Innovación de la Unión Europea (FP7).

Proyecto IntArSu – Interacciones árbol-suelo y recuperación de zonas degradadas. Proyecto Retos. Referencia: CGL2017 – 82254 – R. Financiado por el Ministerio de Ciencia, Innovación y Universidades.

A mis padres, Juan José y Rosario, y a Tom

“Nature seemed to me full of wonders, and I wanted to steep myself in them. Every stone, every plant, every single thing seemed alive and indescribably marvelous. I immersed myself in nature, crawled, as it were, into the very essence of nature and away from the whole human world.”

Carl Gustav Jung

AGRADECIMIENTOS

Quiero agradecer a las cuatro personas más importantes que han hecho posible que esta tesis haya quedado tan maravillosa y por lo bien que me he sentido trabajando con ellos. Gracias Teodoro, Maite, Carmen y Álvaro.

Algo esencial con lo que me quedo de esta experiencia es que mis directores, Teodoro y Maite, son los mejores. Ha sido un auténtico placer poder compartir con vosotros este tiempo. Agradezco la confianza que depositasteis en mí, la libertad y la independencia para poder investigar sobre lo que quisiera, la posibilidad de viajar y crecer profesionalmente, y haberme transmitido vuestros conocimientos.

Teodoro, gracias por guiarme y por preocuparte de mí, por tu serenidad y realismo en este camino. Te deseo lo mejor en tu jubilación, y espero haber sido una buena "última" doctoranda.

Maite, gracias por toda la ayuda, eres una crack, me encanta como trabajas y tu actitud conmigo ha sido más de amiga que de jefa. Gracias por tus consejos en tantos temas. Ya eres una gran investigadora y ahora serás una gran mamá. ¡Enhorabuena!

Carmen y Álvaro habéis sido mis hermanos mayores a los que les preguntaba sin parar y siempre me habéis brindado vuestra ayuda con mucha paciencia. Ambos os habéis implicado muchísimo con esta tesis así que os designo "unofficial directors".

Carmen, has sido mi gran apoyo diario y la que me has enseñado todo eso de la investigación que no sabes cuando empiezas. Llegar todas las mañanas y contarnos nuestras tonterías era un chute de alegría para empezar el día, y lo eché de menos los últimos meses. Te mereces YA una plaza fija, mucha suerte en la oposición.

Álvaro, has sido el que me ha introducido el gusanillo por los hongos y las micorrizas (¡creo que no sabía lo que eran antes de conocerte!). Gracias por dedicar tanto tiempo a enseñarme y repetirme mil veces los conceptos que me costaba entender. También por introducirme y acogerme en Granada y Copenhague… fue una experiencia inolvidable. Deseo que te surjan grandes oportunidades, porque te las mereces todas.

Gracias al resto del Grupo Soilplant, a los investigadores Paula, Engracia, Paco, José Manuel y Rafael por su gran acogida, y a Patri y Cristina. Destacar a Paula y a José Manuel, con los que ha sido un honor y un placer poder colaborar. A José Manuel y José María agradecerles su ayuda en el campo y su alegría. Nunca olvidaré, cuando pinchamos

la furgoneta en mi primer día de campo, lo bien que nos lo pasamos, sin parar de reír ("eso en Madrid no pasa" pensé).

También agradezco al resto de colaboradores que han participado en esta tesis con su conocimiento científico: Mark, Rasmus, Søren y Conchi.

Pero no todo ha sido trabajar… ha habido mucho cachondeo, ya que lo más importante en la vida de un doctorando es tener una gran vida social. Los doctorandos del IRNAS habéis sido mi segunda familia estos años (y espero que así siga siendo por muchos años más). Gracias por tantas fiestas, ferias, salseo, días de playa, campito, juegos de mesa, paintball, desayunos, comidas, tapas y "ven a cenar conmigo", y por lo que nos queda. Os conocí a algunos al principio, otros a mitad y otros al final pero todos vosotros me habéis hecho ser un poquito más feliz: Marta, María del Mar, Carlos, Kike, Josan, Alba, Desi, Marina, Carlos, Paloma, Letizia, Layla, Pro, Sol, Laura, Migue, Elena… y especialmente a los de la tercera planta al final, Lola, por nuestras charlas, y a Pablo Homet, por nuestros quebraderos de cabeza con R; a Pablo Díaz, por ser el alma del IRNAS (y de la fiesta), y a mi María, que tanto echo de menos y que quiero que vuelva a Sevilla. Gracias a la familia IMUS que me ha acogido como una matemática más. No olvidaré las fiestas y los perreos vividos, y espero que lo podamos seguir compartiendo.

Aprovecho para agradecer al resto de amigos que desde lejos de Sevilla me han estado apoyando. A los UAMbientólogos, en especial a Pablo y Ana, los doctores que me comprendieron tan bien, y a María, por estar siempre ahí. A Itziar, mi amiga del alma que siempre querré. Y a mis chicas del pueblo, María, Bea y Paty, que entre nosotras acabamos con todos los problemas.

Agradezco el apoyo incondicional de mi familia, a la que amo profundamente. Mamá, gracias por nuestras conversaciones, por todo el apoyo psicológico y por nuestra conexión. Papá, gracias por tu apoyo, por ser mi fan número 1, leerte todo lo que hago y revisarlo, me hace mucha ilusión que te guste tanto. Gracias a mi hermana Ana, a Juanjo y a mis sobrinos, Juan y Carmen, que sois lo más bonito del mundo y me encanta pasar tiempo con vosotros. Thank you Teana and Ian for your support and love.

El mayor agradecimiento va para el amor de mi vida, Tom. Como tantas veces te he dicho, GRACIAS POR TODO, porque es increíble todo lo bueno que produces en mí y como a tu lado soy cada día mejor persona. Me has ayudado a quererme más, a confiar en mí y a ver que sí podía acabar esta tesis. Gracias por el apoyo en estos cuatro años, en los que hemos vivido tantas aventuras juntos, y por hacerme tan feliz cada día. TE AMO.

RESUMEN

El corredor Verde del Guadiamar se trata de una zona contaminada por elementos traza tras un vertido minero en 1998. Tras este accidente se estableció una estrategia de fitorremediación con el objetivo de remediar y establecer un ecosistema novel.

El primer objetivo de esta Tesis Doctoral fue evaluar la influencia de la identidad de las especies arbóreas forestadas en el Corredor en la composición y actividad microbianas. Se estudiaron los suelos bajo tres especies leñosas (acebuche, álamo blanco y pino piñonero) y en suelos de pradera, en dos sitios con diferentes propiedades edáficas. La forestación produjo un aumento en la mayoría de los indicadores microbianos. Cada especie leñosa produjo efectos específicos en la materia orgánica del suelo, el pH y la relación C:N, con consecuencias en la biomasa y la actividad microbianas. El álamo blanco fue la especie que promovió una mayor diversidad catabólica lo que podría suponer una mayor mineralización de sustratos simples y complejos de carbono en estos suelos. Además, tanto el pino piñonero como el álamo blanco promovieron una mayor actividad enzimática en estos suelos.

El segundo objetivo de esta Tesis Doctoral fue evaluar el efecto de las diferentes especies arbóreas forestadas en el Corredor en la estructura y composición de las comunidades de hongos del suelo. La forestación de suelos degradados con diferentes especies de plantas puede promover el establecimiento de una diversidad y funcionalidad fúngicas determinadas. Se seleccionaron cinco hábitats diferentes: bajo la copa de tres especies leñosas (acebuche, álamo blanco y pino piñonero), en suelos de pradera adyacentes y en suelos no remediados. Encontramos que las medidas de fitorremediación promovieron la riqueza, diversidad, taxonomía y funcionalidad de hongos del suelo en el Corredor Verde del Guadiamar, en comparación con los suelos no remediados. El álamo blanco fue la especie leñosa con la mayor diversidad y riqueza en comparación con el acebuche y el pino piñonero. Los hongos ectomicorrícicos fueron más dominantes en los suelos de las especies hospedadoras de estos hongos, mientras que los saprótrofos fueron abundantes en suelos de pradera y bajo acebuche.

El tercer objetivo de esta Tesis Doctoral fue explorar los cambios funcionales basados en los rasgos de las comunidades de hongos ectomicorrícicos en simbiosis con la encina. La

variación de la composición de las especies ectomicorrícicas estuvo determinada por el C, el Ca y la contaminación del suelo; sin embargo, la diversidad taxonómica no dependió del nivel de contaminación. Los valores de los rasgos medios de las comunidades ectomicorrícicas mostraron una disminución en la formación de rizomorfos e hifas emanentes al aumentar la contaminación, y la comunidad convergió hacia especies con un desarrollo de rizomorfos menos frecuente. Esto supondría una reducción de la diversidad funcional en las comunidades de hongos ectomicorrícicos debido a la contaminación con un efecto potencial en el funcionamiento del ecosistema.

El cuarto objetivo de esta Tesis Doctoral fue evaluar los rasgos funcionales de las especies de hongos ectomicorrícicos. En particular, se estudió cómo la composición de hongos ectomicorrícicos y sus rasgos morfológicos median en los rasgos de la encina. La taxonomía y la diversidad funcional de los hongos ectomicorrícicos explicaron una alta proporción de la varianza de los rasgos funcionales de la encina, tanto en raíces como en hojas. Las encinas que estuvieron dominadas por los hongos más abundantes *Hebeloma cavipes* y *Thelephora terrestris* mostraron unos rasgos conservadores en relación al espectro económico de la raíz, mientras que las encinas colonizadas por especies ectomicorrícicas poco abundantes presentaron una estrategia adquisitiva. Las raíces más conservadoras presentaron unos hongos con elevada formación de rizomorfos y baja melanización. Estos resultados apoyaron el potencial de los hongos ectomicorrícicos, tanto por su taxonomía como por sus rasgos, en la mediación del estado de la planta hospedadora.

El quinto, y último, objetivo de esta Tesis Doctoral fue evaluar la capacidad de los hongos para movilizar los elementos traza del suelo, mediante el análisis de sus cuerpos fructíferos (setas). Se exploraron las relaciones suelo-hongo a través de dos setas silvestres, la especie ectomicorrícica *Laccaria laccata* y la especie saprótrofa *Volvopluteus gloiocephalus*. El análisis isotópico mostró que las setas de *Laccaria laccata* estaban enriquecidas en ^{15}N en comparación con las setas de *Volvopluteus gloiocephalus*, posiblemente por la transferencia de nitrógeno poco enriquecido en ^{15}N a la planta hospedadora. Además, las setas de *Laccaria laccata* mostraron unos valores $\delta^{13}C$ que indicaban que el carbono podría provenir del hospedador mientras que los valores $\delta^{13}C$ de las setas de *Volvopluteus gloiocephalus* fueron similares a los del suelo. Ambas especies mostraron una alta bioacumulación de Cd y Cu en sus cuerpos fructíferos. El consumo humano de estas setas podría representar un riesgo de toxicidad por la elevada concentración de Cd.

ABSTRACT

The Guadiamar Green Corridor is an area contaminated by trace elements due to a mine spill in 1998. After this accident, a phytoremediation strategy was established with the aim of remediating and establishing a novel ecosystem.

The first objective of this Doctoral Thesis was to evaluate the influence of the identity of the forested tree species in the Corridor on the composition and microbial activity. Soils were sampled under three tree species (wild olive, white poplar and stone pine) and in grassland soils, at two sites with different soil properties. Afforestation produced an increase in most microbial indicators. Each tree species produced specific effects on soil organic matter, pH and C:N ratio, with consequences on biomass and microbial activities. White poplar was the species that promoted a greater catabolic diversity, which could mean a greater mineralization of simple and complex carbon substrates in these soils. Furthermore, both stone pine and white poplar promoted greater enzymatic activity in these soils.

The second objective of this Doctoral Thesis was to evaluate the effect of the different afforested tree species in the Corridor on the structure and composition of soil fungal communities. Afforestation of degraded soils with different plant species may promote the establishment of specific fungal diversity and functionality. Five different habitats were selected: under the canopy of three tree species (wild olive, white poplar and stone pine), on adjacent grassland soils and on non-remediated soils. We found that the phytoremediation measures promoted the fungal richness, diversity, taxonomy, and functionality along the Guadiamar Green Corridor, compared to non-remediated soils. White poplar was the tree species with the greatest diversity and richness compared to wild olive and stone pine. Ectomycorrhizal fungi were more dominant in the soils of the host species of these fungi, while saprotrophs were abundant in grassland and wild olive soils.

The third objective of this Doctoral Thesis was to explore the functional changes based on the traits of the ectomycorrhizal fungal communities in symbiosis with the oak. The variation in the composition of ectomycorrhizal species was determined by C, Ca, and soil contamination; however, taxonomic diversity did not depend on the level of

contamination. The values of the mean features of the ectomycorrhizal communities showed a decrease in the formation of rhizomorphs and emanating hyphae with increasing contamination, and the community converged towards species with less frequent development of rhizomorphs. This could mean a reduction in functional diversity in ectomycorrhizal fungal communities due to contamination with a potential effect on the functioning of the ecosystem.

The fourth objective of this Doctoral Thesis was to evaluate the functional features of the ectomycorrhizal fungal species. In particular, how the composition of ectomycorrhizal fungi and their morphological features mediate the features of the holm oak were studied. The taxonomic and functional diversity of ectomycorrhizal fungi explained a high proportion of the variance of the functional features of the holm oak, both in roots and in leaves. The holm oaks that were dominated by the most abundant fungi *Hebeloma cavipes* and *Thelephora terrestris* showed conservative features in relation to the economic spectrum of the root, while the oaks colonized by rare ectomycorrhizal species presented an acquisitive strategy. The most conservative roots presented fungi with high formation of rhizomorphs and low melanisation. These results supported the potential of ectomycorrhizal fungi, both for their taxonomy and for their traits, in mediating the state of the host plant.

The fifth and last objective of this Doctoral Thesis was to evaluate the ability of fungi to mobilize trace elements in the soil, by analysing their fruiting bodies (mushrooms). Soil-fungus relationships were explored through two wild mushrooms, the ectomycorrhizal species *Laccaria laccata* and the sapropharopic species *Volvopluteus gloiocephalus*. Isotopic analysis showed that *Laccaria laccata* mushrooms were ^{15}N-enriched compared to *Volvopluteus gloiocephalus* mushrooms, possibly by transfer of ^{15}N-depleted N to the host plant. Furthermore, *Laccaria laccata* mushrooms showed $\delta^{13}C$ values indicating that C could come from their host, while the $\delta^{13}C$ values of *Volvopluteus gloiocephalus* mushrooms were similar to those in their soil. Both species showed a high bioaccumulation of Cd and Cu in their fruiting bodies. Human consumption of these mushrooms could represent a toxicity risk due to the high concentration of Cd.

ÍNDICE

LISTADO DE TABLAS

LISTADO DE FIGURAS

1. INTRODUCCIÓN

1.1 Degradación y contaminación de suelos

La contaminación y degradación de los suelos es uno de los principales problemas ambientales a escala global. El término "contaminación" se refiere a la presencia de una sustancia o elemento químico en una concentración mayor a las habituales en condiciones normales, mientras que el término "polución" añade que esa sustancia o elemento tiene efectos nocivos en los organismos (Kabata-Pendias y Pendias, 2001; Rodríguez-Eugenio et al., 2018). La mayor parte de los contaminantes provienen de actividades humanas, como la industria, la minería, la agricultura y las actividades domésticas. El sector de la producción industrial es el que más contribuye a la contaminación local del suelo (60% de las zonas contaminadas en Europa), seguido del sector servicios (32% de las zonas), y las actividades mineras (7% de las zonas) (Panagos et al., 2013; van Liedekerke et al., 2014). En Europa, la estrategia temática para la protección del suelo identifica la contaminación local del suelo como un problema importante, y una de las potenciales amenazas para la conservación de los suelos europeos (Comisión de las Comunidades Europeas, 2006). Los últimos datos recogidos en Europa estiman que hay unos 2.5 millones de sitios potencialmente en riesgo de ser contaminados, de los cuales se estima que un 14 % serán contaminados y requerirán de medidas de recuperación o remediación. Los contaminantes más frecuentes son los aceites minerales, los metales pesados, los compuestos orgánicos y los radionucleidos artificiales (European Environment Agency y United Nation Enviromental Programme, 2000; van Liedekerke et al., 2014).

La contaminación produce efectos negativos en la salud y fertilidad de los suelos, además de limitar sus usos. Sin embargo, nuestro conocimiento de los impactos que esta contaminación genera en la biota del suelo y en sus funciones aún es relativamente escasa; apenas se conocen las complejas relaciones que existen entre la contaminación de los suelos y la biodiversidad terrestre (European Environment Agency, 2019).

La restauración completa de la multi-funcionalidad de un suelo que ha sufrido degradación o contaminación es prácticamente imposible y, además, es muy costosa económicamente. Por ejemplo, el coste medio estimado por las administraciones públicas europeas en la gestión de sitios contaminados es de 10€ por habitante por país (van Liedekerke et al., 2014). Las medidas de restauración se centran, fundamentalmente, en estrategias que restauren algunas de las principales funciones de los suelos, tanto

ecológicas (producción de biomasa, filtrado y transformación de sustancias, y hábitat de flora y fauna) como socio-económicas (fuente de materias primas, preservación del patrimonio natural y cultural, y soporte de infraestructuras) (European Environment Agency y United Nation Enviromental Programme, 2000).

La minería y la fundición de los minerales liberan grandes cantidades de metales pesados al medio ambiente que persisten durante largos períodos de tiempo, aumentando sus concentraciones en el medio cada año y generando efectos negativos a largo plazo en los ecosistemas (Ali et al., 2013; Rodríguez-Eugenio et al., 2018).

1.2 Contaminación de suelos por elementos traza y propuestas para la restauración

El término "elementos traza" se refiere a los elementos químicos que se encuentran en el suelo en bajas concentraciones (por debajo de 100 mg kg^{-1}). Existen otros términos como "metales pesados", "metales tóxicos" y "metales traza" que se utilizan como sinónimos, sin embargo, no todos los elementos traza son metales. Por lo tanto es más inclusivo y apropiado utilizar el término "elementos traza". La mayor parte de elementos traza son metales (como Cd, Cr, Co, Cu, Pb, Mn, Hg, Mo, Ni, Tl y Zn), pero también se incluyen metaloides (como B, As y Sb), no metales, actínidos y halógenos (Hooda, 2010).

Los elementos traza se encuentran de manera natural en los suelos. Algunos de estos elementos son micronutrientes esenciales para los seres vivos, sin embargo, en altas concentraciones pueden causar problemas de toxicidad, bioacumulación y persistencia, lo cual depende de sus propiedades, especiación y niveles de concentración (Kabata-Pendias y Pendias, 2001). Los elementos traza más tóxicos para los humanos y animales son Hg, Pb, Cd, Cr y As; mientras que para las plantas son Cu, Ni, Co y Zn (Rodríguez-Eugenio et al., 2018).

Los elementos traza acumulados en el suelo pueden reubicarse lentamente en el ecosistema por medio de la lixiviación, erosión y absorción en plantas. El comportamiento y la disponibilidad de los elementos traza para los organismos vivos dependen de su especiación (estructura química), su concentración, el tipo de suelo, la vegetación y el clima. Las propiedades del suelo son especialmente importantes en la dinámica de los elementos traza. La solubilidad de los elementos traza suele explicarse

en función del pH del suelo y de la cantidad y calidad de la materia orgánica, influyendo también la capacidad de intercambio catiónico, el contenido en carbonatos y óxidos e hidróxidos de Fe y Mn, así como la textura (contenido en arcillas y fracciones finas) (Kabata-Pendias y Pendias, 2001). Los elementos traza son considerados los contaminantes más persistentes en el suelo por su tendencia a acumularse y por el riesgo de introducirse en la cadena trófica. A diferencia de otros tipos de contaminación, los elementos traza no pueden ser biodegradados, y la gestión debe centrarse en recuperarlos del suelo o en inmovilizarlos en la propia matriz del suelo (Kumpiene et al., 2019).

Un suelo contaminado por elementos traza puede gestionarse de diversas maneras. Las técnicas de recuperación pueden ser *in situ* (en el sitio de la contaminación) o *ex situ* (implicando el traslado del suelo contaminado). Se pueden llevar a cabo diferentes tratamientos físicos, químicos o biológicos para tratar de reducir su concentración, reducir su biodisponibilidad, encapsular, contener o eliminar (Rodríguez-Eugenio et al., 2018). Una de las gestiones *ex situ* es la de excavar el suelo contaminado, trasladarlo a un vertedero y reemplazarlo con suelo limpio. Esta es la técnica de remediación más utilizada en Europa, aplicada en un 30% de las áreas contaminadas (van Liedekerke et al., 2014). Otra de las opciones es excavar el suelo, descontaminarlo y depositarlo de nuevo en su ubicación original. Este tipo de gestiones *ex situ* que se centran en un tratamiento físico tienen varios inconvenientes. El primero es que en áreas con grandes extensiones afectadas, como pueden ser las zonas mineras, esta opción no es viable económicamente. El segundo es que el traslado y acumulación de suelo contaminado en otros enclaves puede generar problemas de lixiviado de contaminantes desde las zonas habilitadas como vertedero a otros ecosistemas y afectar a los organismos, incluidos humanos (Kumpiene et al., 2019). Es necesario, por tanto, aplicar métodos de recuperación más eficientes, sostenibles y de bajo coste para la descontaminación de suelos contaminados por elementos traza (Ali et al., 2013).

La fitorremediación es una técnica *in situ* que genera numerosas ventajas y tiene un elevado potencial para restaurar la funcionalidad del ecosistema (Garbisu et al., 2002). Se trata de una remediación natural asistida que juega un importante papel en la restauración de las propiedades físicas, químicas y biológicas de los suelos contaminados (Clemente et al., 2006). Esta técnica incluye tecnologías que utilizan las plantas y sus microorganismos asociados para descontaminar y revegetar los suelos contaminados (Bolan et al., 2011). La fitorremediación es considerada la tecnología más factible para recuperar grandes extensiones contaminadas por elementos traza debido a su bajo coste

(en instalación y en mantenimiento) y a la mejora estética del paisaje que puede proporcionar (Ali et al., 2017, 2013; Mendez y Maier, 2008). Las plantas tienen una gran capacidad para retener contaminantes del suelo, evitando la transferencia de elementos traza a otros sistemas adyacentes y proporcionan beneficios a largo plazo, aumentando la funcionalidad del ecosistema (Madejón et al., 2018a). Las plantas conservan el suelo superficial, mantienen su uso, reducen la erosión y el lixiviado, y aumentan la fertilidad del suelo al ser una fuente de materia orgánica (Ali et al., 2013).

Para desarrollar la máxima funcionalidad a largo plazo en el ecosistema restaurado, es esencial el establecimiento y supervivencia de la vegetación. Es importante por tanto seleccionar las especies vegetales más apropiadas para las condiciones específicas de la zona contaminada. En general, la selección de especies nativas ayuda a la supervivencia, crecimiento y reproducción de las plantaciones, ya que están adaptadas al suelo y al clima local. También es condición previa que las propiedades físico-químicas del suelo sean adecuadas para el crecimiento vegetal, lo cual puede conseguirse mediante el pre-tratamiento del suelo con la aplicación de enmiendas para la reducción de los niveles de toxicidad (Kumpiene et al., 2019; Zhou et al., 2015). Desarrollar estos ecosistemas "noveles" o emergentes puede requerir coste y trabajo extra, como el uso de riego, fertilización o control de plagas. Con ellos se evita de manera activa que se creen ecosistemas de baja diversidad o inestables (Wójcik et al., 2014).

Aunque el establecimiento de una cobertura vegetal puede conllevar numerosos beneficios, también hay que atender a posibles efectos no deseados, por ejemplo, exudados de moléculas orgánicas que alteran el pH del suelo aumentando la movilidad y absorción por las plantas de elementos traza. Asimismo, las comunidades microbianas, libres en el suelo o bien asociadas a las plantas, también pueden alterar estos procesos (Kumpiene et al., 2019).

1.3 Diferentes estrategias de fitorremediación

Existen tres enfoques de fitorremediación que son los más utilizados en áreas contaminadas por elementos traza: fitoextracción, fitovolatilización y fitoestabilización.

La "fitoextracción" es el uso de plantas que concentran elementos traza en su biomasa aérea, de manera que los metales acumulados puedan ser retirados del sistema mediante la cosecha de esta biomasa. Para este tipo de remediación se utilizan plantas con

estrategias acumuladoras o hiperacumuladoras, que pueden acumular hasta 100 veces mayor concentración de elementos fitotóxicos en su biomasa aérea que las plantas no acumuladoras (Pulford y Watson, 2003; Wójcik et al., 2014). Estas especies tienen la capacidad de movilizar elementos traza de las fracciones poco solubles del suelo y acumularlos en la biomasa aérea. Algunos de los inconvenientes de esta estrategia se deben a que la recuperación está limitada por la lenta velocidad de crecimiento de estas especies hiperacumuladoras, que suelen producir poca biomasa. Además, hay algunos elementos traza que tienen baja movilidad, como el Pb, para el que no se conocen especies hiperacumuladoras (Robinson et al., 2003). Tras la acumulación de elementos traza, la biomasa vegetal es potencialmente peligrosa (por su toxicidad) y las plantas deben ser cosechadas y sacadas fuera de la zona recuperada. Esta biomasa rica en elementos traza puede ser quemada, fermentada o gasificada para reducir su volumen; los residuos generados podrían ser procesados para recuperar los elementos valiosos o ser almacenados de manera segura (Robinson et al., 2009).

La "fitovolatilización" es la transformación de los contaminantes en compuestos volátiles que se dispersan en la atmósfera a través de las plantas. Los elementos que pueden volatilizarse a través de algunas plantas están limitados a tres: As, Hg y Se. Su mayor inconveniente es que no se puede controlar el destino de los elementos volatilizados, pero cuenta con la ventaja de que no es necesario cosechar la biomasa (Robinson et al., 2009).

La "fitoestabilización" persigue la reducción de la movilidad y biodisponibilidad de los contaminantes en el medio, mediante la inmovilización en las raíces de las plantas o por prevención de la migración mediante su precipitación en la rizosfera (Kumpiene et al., 2019; Pulford y Watson, 2003). Entre sus ventajas se encuentra la particularidad de que no es necesario mover los contaminantes del sitio afectado, y que no genera residuos secundarios que necesiten tratamiento. Sin embargo, el suelo es un sistema en continuo cambio donde se producen procesos de inmovilización-movilización de los elementos traza (Kabata-Pendias y Pendias, 2001). Es esencial monitorizar y controlar que las condiciones de estabilidad se mantienen, adoptando las decisiones de manejo en base a este seguimiento de manera que podría ser necesaria la aplicación periódica de enmiendas para mantener los contaminantes inmovilizados en el suelo (Bolan et al., 2011). La aplicación de enmiendas, por lo general, induce cambios químicos en el suelo que reduce la disponibilidad de estos elementos traza para la biota, favoreciendo el proceso de estabilización de los elementos traza en el suelo Las enmiendas más aplicadas en el

contexto de fitoestabilización son, dependiendo del tipo de contaminación, materiales orgánicos compostados, biocarbón (biochar), materiales con Fe, cenizas y fosfatos.

El potencial remediador de las plantas, en función de los tres enfoques de fitorremediación mencionados, se basa en su contribución a la transferencia de metales y en su adecuación como evaluadoras del riesgo o bioindicadores. Las diferentes especies vegetales pueden desarrollar tres estrategias en relación con la concentración de elementos traza en el suelo, clasificándose como "acumuladoras" (mayor concentración en la planta que en el suelo; adecuadas para la fitoextracción), "exclusoras" (menor concentración en la biomasa aérea que en el suelo; adecuadas para la fitoestabilización), e "indicadoras" (correlación entre planta y suelo; adecuadas para conocer la biodisponibilidad de elementos en el suelo) (Wójcik et al., 2014). Es esencial conocer la estrategia de las especies potenciales para la restauración y seleccionarlas adecuadamente, según la estrategia de fitorremediación que interese aplicar. La fitoestabilización reduce el riesgo de transferencia de elementos traza a través de la cadena trófica si se seleccionan especies que inmovilicen los metales en la rizosfera y que no acumulen estos elementos en la biomasa aérea (Bolan et al., 2011). La implementación de un programa de fitoestabilización puede considerarse como un proceso con tres etapas: primero, la selección y aplicación de enmiendas según el tipo y nivel de contaminación; segundo, la plantación de las especies vegetales más adecuadas; y tercero, la monitorización en el tiempo de los cambios en la movilidad de los elementos traza en el sistema (Bolan et al., 2011; Domínguez et al., 2008; Madejón et al., 2018a) (Fig. 1.1).

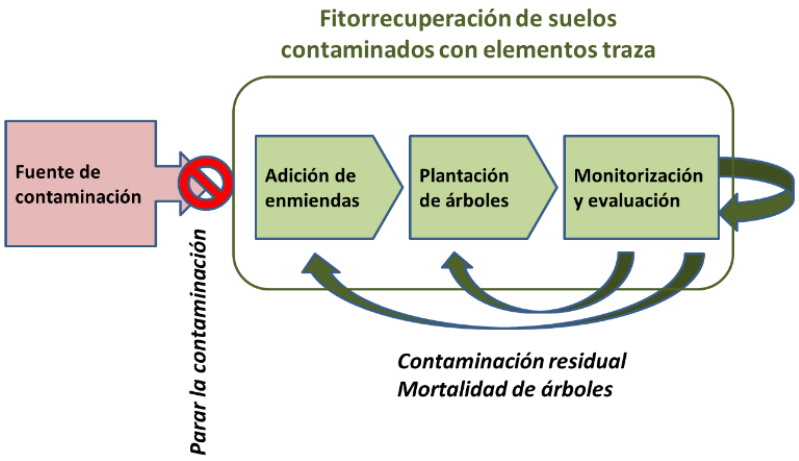


Figura 1.1 Esquema del proceso de fitoestabilización con plantaciones de árboles en suelos contaminados por elementos traza (tomado de Madejón et al. (2020)).

1.4 Las interacciones planta-suelo en un escenario de fitorremediación

El término "interacción planta-suelo" hace referencia a los cambios que las plantas realizan en las propiedades bióticas y abióticas del suelo y, a su vez, al efecto que estos cambios tienen en la nutrición y desarrollo de las plantas (van der Putten et al., 2013). No se conoce bien de qué manera las interacciones planta-suelo afectan al ensamblaje del ecosistema, pero sabemos que son importantes para la restauración ecológica (Eviner y Hawkes, 2008; Harris, 2009; Kardol y Wardle, 2010; van der Putten et al., 2013). Gracias al desarrollo de técnicas moleculares, en los últimos años se han estudiado las interacciones entre las plantas y las comunidades microbianas del suelo, para descubrir el papel de la microbiota en la diversidad y dinámica de las comunidades vegetales (Bever, 2003; Erktan et al., 2018; Rutten y Gómez-Aparicio, 2018; van der Heijden et al., 2015; Wardle et al., 2004).

Los cambios físicos, químicos y biológicos que se producen en el suelo tras la forestación conllevan importantes efectos en la funcionalidad del ecosistema (Aponte et al., 2013; Grosso et al., 2018; Mitchell et al., 2010a). Las comunidades vegetales tienen importantes efectos en el suelo, mediante los efectos de rizodeposición y de la descomposición de la hojarasca y las raíces muertas (Nannipieri et al., 2017). Cuando la vegetación empieza a desarrollarse en las zonas contaminadas y restauradas, la materia orgánica se acumula de manera natural en el suelo, afectando a la relación entre las plantas, los microbios y los elementos traza. La materia orgánica juega un papel fundamental debido a su relevancia en el ciclado de nutrientes y a su capacidad para secuestrar o adsorber metales (Bolan et al., 2011; Brookes, 1995). En la materia orgánica se genera un ciclo de adsorción – liberación de estos metales, el cual depende de la calidad de la materia orgánica y de su dinámica de descomposición, generando un sistema dinámico complejo que se mueve entre fracciones estables y biodisponibles de estos elementos (Krumins et al., 2015).

No todas las especies de plantas producen los mismos efectos en las comunidades microbianas del suelo. Por ejemplo, las especies de árboles difieren en la profundidad y estructura de sus raíces, en las asociaciones simbióticas que establecen con los microorganismos del suelo, en el transporte radicular y en su habilidad para movilizar nutrientes (Carnol y Bazgir, 2013). De manera directa, la composición química y el contenido en nutrientes de los tejidos vegetales determinarán la tasa de descomposición

realizada por los microbios del suelo. De manera indirecta, las especies dominantes de plantas pueden modificar propiedades abióticas como el pH, el contenido en materia orgánica y la estructura del suelo. También pueden mediar en procesos biogeoquímicos, como los de acidificación o nitrificación (Angst et al., 2018; Berg y McClaugherty, 2014; Madejón et al., 2018a; Madejón et al., 2018b; Marañón et al., 2015; Orozco-Aceves et al., 2015) que acaben afectando a las comunidades microbianas del suelo (Prescott y Grayston, 2013). En plantaciones forestales, como las realizadas para la fitorremediación de suelos contaminados, los efectos de estas especies en el funcionamiento ecosistémico serán diferentes, dependiendo si las especies son plantadas en monocultivos o en plantaciones mixtas, y dependiendo de la cantidad y calidad de los aportes vegetales al suelo (Richards et al., 2010).

Por otro lado, las plantas dependen en gran medida del microbioma de la rizosfera para la absorción de nutrientes, la protección frente a diferentes fuentes de estrés biótico y abiótico, y la mejora de la arquitectura radicular. Las plantas parecen haber co-evolucionado con los microbios del suelo para adoptar estrategias de vida que beneficien tanto a las funciones de la planta como al desarrollo del microbioma radicular (Bakker et al., 2018). Por lo tanto, la influencia de la vegetación en el funcionamiento del ecosistema esta mediada en buena parte por la biota de la rizosfera (Friesen et al., 2011). Por ejemplo, las interacciones entre las raíces de las plantas y los organismos del suelo pueden dar lugar a alteraciones en el crecimiento y en la calidad nutricional de la biomasa aérea vegetal. Los cambios producidos por la biota del suelo en las raíces pueden inducir cambios en la concentración de los compuestos químicos en las hojas. Si se modifica la calidad de los tejidos vegetales, se modifican entonces las interacciones multitróficas de las partes aéreas y subterráneas. Por lo tanto, las relaciones planta-suelo tienen el potencial de modificar los procesos de selección y evolución vegetal (van der Putten et al., 2013). A la vez, debido a la retroalimentación (*feedback*) entre las plantas y los microorganismos del suelo, las comunidades de plantas y sus rasgos funcionales modifican la estructura de la composición microbiana del suelo (Aponte et al., 2013; Bauman et al., 2016; de Vries et al., 2012; López-García et al., 2017).

En suelos contaminados por elementos traza, los microorganismos y los procesos que éstos realizan pueden verse afectados, produciendo graves perturbaciones en los ecosistemas (Clarholm y Skyllberg, 2013a; Giller et al., 1998; Krumins et al., 2015). Concentraciones elevadas de elementos traza, debido a su toxicidad, reducen la biomasa microbiana del suelo, pudiendo limitar, además, la eficiencia de las comunidades

microbianas en la utilización de los sustratos (Chander y Joergensen, 2001) y la producción de enzimas (Kandeler et al., 2000), causar cambios en la diversidad y estructura de estas comunidades (Abaye et al., 2005), o bien, provocar todos estos efectos conjuntamente (Khan et al., 2010).

La restauración de ecosistemas tras un evento de contaminación por elementos traza requiere el restablecimiento de las interacciones positivas entre las plantas y las comunidades microbianas del suelo (Krumins et al., 2015). Es de vital importancia estudiar los efectos de estos elementos traza en el ecosistema para poder aplicar medidas de restauración adecuadas en estas áreas degradadas. En lo que a fitorremediación se refiere, existe una falta de conocimiento suficiente sobre los efectos de esta técnica en los ecosistemas, así como de la capacidad de restauración de diferentes especies de plantas y los efectos en diferentes tipos de suelos. Hasta la fecha, la fitorremediación se ha centrado principalmente en determinar qué especies de plantas son capaces de acumular o excluir metales, pero en numerosas ocasiones la habilidad de la planta para tolerar elementos traza está directamente relacionado con los organismos presentes en su rizosfera (Kuiper et al., 2004). Las respuestas de las plantas y los organismos del suelo son interdependientes y dinámicas, pero la dimensión en la que la comunidad de plantas afecta a las comunidades del suelo y viceversa es aún relativamente desconocida (van der Bij et al., 2018).

En resumen, las interacciones planta-suelo son esenciales para el desarrollo del ecosistema tanto en suelos contaminados como en suelos saludables. Sin embargo, en el caso de los suelos contaminados hay que incluir a los elementos traza dentro de estas interacciones. Por lo tanto, existen cuatro componentes que van a determinar estas interacciones en sistemas contaminados: comunidades microbianas, comunidades de plantas, propiedades abióticas del suelo y elementos traza (Fig. 1.2), produciéndose respuestas bidireccionales y multidirecccionales entre todos los componentes.

Figura 1.2 Esquema de los cuatro componentes de estudio en interacciones planta-suelo en suelos contaminados por elementos traza (ilustración propia).

1.5 El suelo como hábitat para los microorganismos: factores abióticos que determinan la actividad y diversidad de la microbiota

El suelo es un sistema extremadamente complejo que contiene una gran amplitud de condiciones ambientales y una variedad de características abióticas. La calidad de un suelo es la capacidad que éste tiene para funcionar dentro de los límites del ecosistema y de interactuar positivamente con los ecosistemas adyacentes. La calidad se evalúa a través de numerosos indicadores físicos, químicos y biológicos, y sus interacciones (Fig. 1.3).

Esta calidad reconoce de manera explícita el papel esencial de la biota del suelo y las funciones que realiza. Existen numerosos indicadores que determinan las características de las comunidades microbianas, que pueden utilizarse para evaluar el éxito de las medidas de restauración (Harris, 2003; Muñoz-Rojas, 2018).

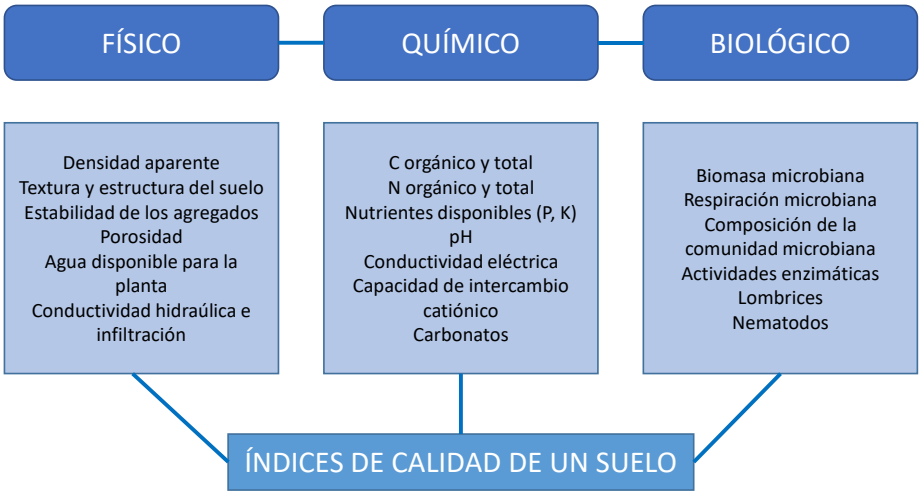


Figura 1.3 Listado de algunos indicadores físicos, químicos y biológicos que se analizan para establecer índices y así conocer la calidad de un suelo (adaptado de Muñoz-Rojas (2018)).

En el suelo se producen unos mecanismos de transporte de sustratos muy complejos, además se generan infinidad de interacciones internas y externas que determinan la estructura y funcionalidad de la comunidad microbiana (Bissett et al., 2013; Fierer, 2017). Dentro de la gran diversidad de vida existente en los suelos, el grupo dominante de organismos, en términos de número y biomasa, está representado por los microorganismos. La identidad y la funcionalidad de los diferentes microorganismos del suelo es muy variable, ocupando diferentes nichos tróficos (Krumins et al., 2015). Los microorganismos necesitan condiciones favorables para dividirse y crecer de manera activa (Berg y McClaugherty, 2014a). Su supervivencia y crecimiento están limitados en el suelo por los factores de estrés abióticos, la competencia entre especies, las frecuentes perturbaciones y la distribución desigual de los recursos en el espacio y el tiempo (Fierer, 2017).

Los microorganismos del suelo dependen en gran medida de la calidad y la estabilidad de la materia orgánica, evaluada por la abundancia y variedad en la complejidad de sustancias orgánicas. Los residuos de las plantas son la principal entrada de materia orgánica primaria en el suelo; la calidad y cantidad de estos residuos influirá en la naturaleza, tamaño, composición, función y propiedades fisiológicas de la microbiota (Berg y McClaugherty, 2014a). Los restos vegetales son un conjunto de compuestos orgánicos complejos, principalmente polisacáridos y lignina, pero también biopolímeros

alifáticos y taninos (Kögel-Knabner, 2002). La composición y abundancia relativa de estos compuestos varía según la especie vegetal y el tipo de tejido. La cantidad, composición y propiedades de los restos vegetales son factores que controlan la formación de la materia orgánica del suelo y los procesos de humificación (Berg y McClaugherty, 2014a). Estos compuestos se descomponen en sustancias orgánicas más simples, que son mineralizadas por los microorganismos y son transformadas a moléculas inorgánicas. Los restos vegetales de alta calidad suelen promover el uso eficiente del carbono, la biomasa y el crecimiento de los microorganismos (Córdova et al., 2018). En el suelo también se producen procesos de humificación. La fracción húmica de la materia orgánica del suelo está compuesta por las sustancias húmicas que tienen estructuras complejas y son resistentes a la biodegradación. Por lo tanto, el humus es una fuente de carbono y de energía de liberación lenta. Los procesos de mineralización y acumulación en la matriz mineral del suelo son, por lo tanto, críticos para la funcionalidad del ecosistema (Córdova et al., 2018). Existen numerosas interacciones entre los organismos que se encargan de la descomposición que van cambiando en el tiempo, según las propiedades de los residuos y los cambios estacionales (Berg y McClaugherty, 2014a).

Aparte de la materia orgánica, las condiciones físico-químicas del suelo que más determinan las comunidades microbianas son el pH, la disponibilidad de nutrientes, el microclima (incluyendo la temperatura y la humedad) y la textura (Aksoy et al., 2017; Chai et al., 2019; Courty et al., 2018; Fierer et al., 2009; Fierer, 2017; Schappe et al., 2017; Vasco-Palacios et al., 2020; Wang et al., 2019). En cuanto al pH del suelo, los microorganismos, por lo general, no toleran pH extremos; aparte de los efectos directos en sus proteínas y enzimas, indirectamente el pH cambia las formas químicas, la solubilidad y disponibilidad de los compuestos químicos. La concentración y biodisponibilidad de nutrientes es esencial para los microbios, ya que N, P y S son los principales elementos limitantes del crecimiento microbiano (Berg y McClaugherty, 2014a). Tanto la humedad como la temperatura afectan a la velocidad de los procesos biológicos, siendo el agua un componente esencial en su crecimiento. En conclusión, las comunidades microbianas del suelo pueden ser modeladas dentro de un contexto de interacciones múltiples de las diferentes propiedades del medio, tanto de manera directa e indirecta, afectando a su composición y función (Waldrop et al., 2017).

Debido a la elevada heterogeneidad del suelo, se pueden encontrar diferentes tipos de hábitats microbianos (Fierer, 2017). Uno de ellos es la “rizosfera”, que es la delgada región (de unos pocos mm de espesor) del suelo que está directamente influenciada por

las raíces de las plantas. Los exudados radicales de las plantas proporcionan nutrientes a los microbios de la rizosfera, aumentando su actividad, la cual estimula el crecimiento vegetal (Khan, 2005). La rizosfera es, por tanto, muy importante en las interacciones planta-microorganismo ya que en ese microhábitat se da una gran proliferación microbiana, produciéndose la mayor parte del flujo y distribución del carbono y el ciclado de nutrientes (Balestrini et al., 2015). Además, en la rizosfera los microorganismos se encuentran metabólicamente muy activos ya que las limitaciones de nutrientes son por lo general menores que en los ambientes más alejados de las raíces.

1.6 El estudio de la microbiota como indicador de la calidad en suelos degradados

Los microorganismos edáficos facilitan muchos procesos de descomposición y mineralización, ciclado de nutrientes y eliminación de contaminantes, además de ser causa y remedio de un rango de enfermedades de las plantas (Uroz et al., 2019).

Los microorganismos del suelo poseen una variedad de adaptaciones evolutivas y mecanismos de aclimatación fisiológica que les permiten sobrevivir y mantenerse activos cuando sufren alguna perturbación ambiental. Sin embargo, estas estrategias generan gastos fisiológicos a nivel de organismo y pueden alterar la composición de la comunidad microbiana activa, produciendo cambios a nivel de ecosistema en los ciclos de carbono, energía y nutrientes (Schimel et al., 2007). Existen numerosos procesos de degradación en el suelo, como la erosión, salinización, pérdida de fertilidad, compactación y contaminación, que pueden modificar la microbiota, impactando negativamente en el funcionamiento del ecosistema. Son numerosos los factores que influyen en la estabilidad de las comunidades del suelo cuando se produce una perturbación, por lo tanto, las respuestas son difíciles de predecir (Griffiths y Philippot, 2012). Una perturbación implica un cambio, a veces drástico, en las condiciones ambientales y una respuesta en las comunidades microbianas, que son interesantes de estudiar. Los microorganismos son indicadores sensibles y rápidos de la funcionalidad del suelo tras un evento de perturbación, debido a su susceptibilidad a los cambios (Nannipieri et al., 2017). Tras un suceso estresante podemos evaluar los efectos inmediatos, pero también podemos evaluar

el progreso de la recuperación natural o asistida a medio y largo plazo, mediante análisis de la diversidad y actividad microbianas (Banning et al., 2011; Bünemann et al., 2018).

Los microbios del suelo son un grupo muy variado en términos de taxonomía, estructura y función (Harris, 2009). La diversidad microbiana en un suelo puede medirse en diferentes niveles, desde la comunidad hasta la estructura y funcionalidad. Por ejemplo, las actividades enzimáticas son muy sensibles a procesos estresantes y perturbadores, por lo tanto, son considerados indicadores eficaces de los cambios funcionales del suelo (Quilchano y Marañón, 2002).

Debido a la complejidad en el estudio de la microbiota, es necesario un enfoque amplio que integre la estructura, la velocidad de los procesos, las fluctuaciones temporales, las interacciones y los procesos de retroalimentación (*feedback*) de las comunidades microbianas (Harris, 2009). Los microorganismos del suelo se están estudiando en relación a la restauración de suelos degradados desde dos vertientes: primero, para indicar el estado de recuperación de un ecosistema comparándolo con un sitio o condiciones de referencia y, segundo, para manipular el sistema con la finalidad de aumentar la velocidad de cambio hacia un estado deseado, superando las barreras bióticas (Harris, 2009). Las formas de superar algunas de estas barreras bióticas podrían ser mediante el aumento de las especies mutualistas, o aumentando la proporción de biomasa fúngica con respecto a biomasa bacteriana (van der Wal et al., 2006).

1.7 Diversidad de hongos y su importancia en la recuperación de suelos contaminados

Generalmente, los hongos son considerados el grupo microbiano más importante debido a sus importantes funciones en la descomposición de la materia vegetal, la exploración del suelo en busca de recursos de los que pueden favorecerse las plantas, y por la producción de las enzimas que degradan la materia orgánica (Berg y McClaugherty, 2014a; Schneider et al., 2012). También, constituyen la mayor fracción de la biomasa viva y muerta del suelo y producen la mayor actividad en los horizontes orgánicos, particularmente en bosques (Taylor y Sinsabaugh, 2015). Son esenciales en los ciclos del C, N y P, ya que son los mayores descomponedores de la materia orgánica de origen vegetal (Balestrini et al., 2015). Debido a su variedad de hábitos de crecimiento, su

potente actividad enzimática y metabolismo secundario, pueden degradar un amplio rango de recursos, incluidos sustratos complejos (Thorn y Lynch, 2006).

La mayoría de los hongos forman unos filamentos llamados hifas que pueden unirse formando una red de filamentos, llamada micelio. Los micelios pueden ser indiferenciados u organizarse en cuerpos fructíferos, como las conocidas setas o trufas.

Especialmente durante las primeras etapas de la descomposición, el micelio puede crecer alrededor de la estructura celular de los residuos vegetales y excretar una variedad de enzimas, como glucosidasas y oxidasas (Taylor y Sinsabaugh, 2015).

A través del micelio, los hongos individuales, las especies y las comunidades fúngicas pueden dominar el suelo, transfiriendo nutrientes y agua entre distancias macroscópicas, interconectar organismos de diferentes niveles tróficos, persistir en el tiempo y ordenar los recursos necesarios para formar los cuerpos fructíferos o setas (Taylor y Sinsabaugh, 2015; Thorn y Lynch, 2006). Mediante sus micelios, los hongos favorecen la creación y estabilización de los agregados del suelo, que son fundamentales en la estructura del suelo (Harris, 2009). La formación de agregados promueve el secuestro de C, al proteger las partículas orgánicas de los descomponedores y las enzimas degradadoras (Wilson et al., 2009). Adicionalmente, la tasa de recambio de la biomasa fúngica es muy importante en el ciclo del C y en su secuestro en el suelo a largo plazo (Clemmensen et al., 2015, 2013; Langley y Hungate, 2003). Debido a estos procesos, el desarrollo de la comunidad fúngica es sumamente importante en la evolución de suelos degradados, en los que tanto la fertilidad química como la estabilidad física pueden encontrarse bastante deterioradas.

Los hongos poseen gran variedad de hábitos de vida, influyendo en diversas funciones ecológicas (Sun et al., 2017). Los hábitos de los hongos se clasifican en 1) saprófitos o de vida libre, con un papel importante en procesos de descomposición, 2) mutualistas, los cuales proveen de múltiples servicios a las plantas terrestres en sus formas de endófitos y micorrizas, y 3) patógenos que dañan a las plantas y regulan la composición de las comunidades vegetales (Naranjo-Ortiz y Gabaldón, 2019; Nilsson et al., 2019; Tedersoo et al., 2014; van der Heijden et al., 2008). Los hongos interaccionan con numerosos organismos, vivos y muertos, y especialmente con las plantas, influyendo en la estructura y la función de las comunidades vegetales (Taylor y Sinsabaugh, 2015). Dependiendo de la especie vegetal con la que interactúa y las condiciones ambientales, la relación hongo-raíz puede variar a lo largo del continuo saprotrofia - biotrofia (Koide et al., 2008).

Los mecanismos moleculares que se producen en las interacciones planta – hongo todavía no son del todo conocidos y se sigue investigando su papel. La biodiversidad del suelo está íntimamente relacionada a las funciones biológicas que se desarrollan en ese suelo; por tanto, investigar los factores ambientales que influyen en las comunidades de hongos es esencial para la caracterización ecológica de un ecosistema (Orgiazzi et al., 2015). La biomasa fúngica del suelo varía ampliamente en relación a la composición de la hojarasca, la densidad radicular y la disponibilidad de nutrientes. Del mismo modo, los hongos están regulados por la cantidad y calidad de la materia orgánica. La cantidad de biomasa fúngica en muchos suelos excede la biomasa del total del resto de organismos del suelo, con excepción de las raíces. Este predominio de los hongos suele suceder en condiciones ambientales determinadas, como la baja concentración de nutrientes, sequía, bajas temperaturas o baja calidad de la hojarasca, cuando la tasa de recambio de la hojarasca y el ciclado de nutrientes son reducidos (Thorn y Lynch, 2006).

Las especies que componen las comunidades de hongos pueden estar influenciadas por numerosos factores abióticos. A macroescala geográfica y temporal, las condiciones climáticas son el mayor predictor de la riqueza y la composición de hongos (Tedersoo et al., 2014). En cuanto a la microescala espacial, los estudios de las comunidades de hongos en diferentes horizontes muestran cómo las especies tienen diferentes preferencias de nicho en el heterogéneo sistema del suelo (Moll et al., 2016). En los horizontes orgánicos superficiales donde hay más abundancia de raíces finas, los nutrientes son abundantes y los procesos de descomposición y mineralización son más intensos (Courty et al., 2008). Sin embargo, debido a la partición de nichos entre especies y gremios de hongos, la abundancia y la composición de especies se distribuye a lo largo del perfil (Clemmensen et al., 2013; Landeweert et al., 2003; Lindahl et al., 2007; Taylor et al., 2010; Tedersoo et al., 2003). Los factores edáficos tienen repercusiones directas e indirectas en las comunidades de hongos y los principales reguladores son el pH, la humedad y las concentraciones de los principales nutrientes, como el N, el P, el Ca y la relación C: N (Courty et al., 2018; Cox et al., 2010; Fierer et al., 2009; Joergensen y Wichern, 2008; Maghnia et al., 2017; Narendrula-Kotha y Nkongolo, 2017; Rousk et al., 2009; Sun et al., 2016; Taylor y Sinsabaugh, 2015; Tedersoo et al., 2014; Vasco-Palacios et al., 2020).

La composición de las comunidades vegetales tiene un papel dominante en la determinación de las comunidades de hongos del suelo (Taylor y Sinsabaugh, 2015). Es de esperar que, según la comunidad de plantas en un sitio determinado, encontremos una

comunidad de hongos específica que tenga preferencias por asociarse a esas especies vegetales. Esto suele ocurrir para esos hongos que se asocian de manera más específica a las plantas, como los hongos micorrícicos, los patógenos de plantas y los endófitos (Fierer, 2017). Las especies leñosas, en comparación con las especies herbáceas, producen mayores cantidades de materia orgánica y sus sistemas radiculares son más extensos, produciendo ambientes más heterogéneos en el suelo (Sun et al., 2016). La principal diferencia encontrada en las comunidades fúngicas entre bosques y praderas es la dominancia de las comunidades ectomicorrícicas en los bosques (Sun et al., 2016; Tedersoo et al., 2012, 2014). Sin embargo, la composición de las comunidades fúngicas es muy variada en bosques; el establecimiento de diferentes especies conllevará una diversidad fúngica asociada a cada una de las especies (Unterseher et al., 2008) o asociada al tipo de vida o de simbiosis micorrícica de estas plantas (Tedersoo et al., 2014). Se han observado cambios en la composición, en la taxonomía y funcionalidad fúngica con la sucesión de las comunidades vegetales, así como en el progreso de descomposición de la hojarasca y de la materia orgánica del suelo (Lindahl et al., 2007; Peršoh, 2015; Taylor et al., 2010; Voříšková y Baldrian, 2013). Asimismo, en un estudio de Cornelissen et al. (2001) se constató que el tipo de hongo micorrícico con el que se asocia cada planta está relacionado con la variación de los rasgos morfológicos relacionados con el ciclo del carbono en la planta. De esta manera, la asociación simbiótica entre las plantas y las micorrizas son relevantes a nivel ecosistémico en los ciclos del carbono y nutrientes. La competencia entre las especies de hongos también estructura estas comunidades fúngicas (Kennedy, 2010). Por ejemplo, el orden de llegada de las diferentes especies ectomicorrícicas puede cambiar la dominancia en la colonización de los sistemas radiculares (Kennedy et al., 2009).

1.8 El papel de los hongos micorrícicos en la fitorremediación

La mayoría de las plantas terrestres forman interacciones simbióticas beneficiosas en sus raíces con hongos, formando las micorrizas. Los hongos micorrícicos son esenciales en los ecosistemas terrestres debido a su influencia tanto en la multifuncionalidad del suelo como en la de todo el ecosistema (van der Heijden et al., 2015). Los hongos micorrícicos regulan los ciclos de carbono y nutrientes mediante la transferencia de nutrientes minerales y agua hacia la planta, a cambio de obtener compuestos de carbono que fija la planta mediante la fotosíntesis (Cornelissen et al., 2001). La planta puede llegar a invertir

un 10 – 30 % de su producción primaria neta en la micorriza. De manera recíproca, aproximadamente el 80 % del N y P que llega a la planta es provisto por los hongos micorrícicos (van der Heijden et al., 2015). Por lo tanto, las micorrizas son los órganos principales de la absorción de nutrientes de las plantas terrestres, dependiendo de esta simbiosis para su supervivencia y crecimiento (Averill et al., 2014; Smith y Read, 2008; Soudzilovskaia et al., 2015). Esta absorción se produce a través de las hifas que crecen desde la micorriza. Además, los hongos micorrícicos tienen la ventaja, sobre los hongos saprófitos, que al ser independientes de las fuentes de carbono del suelo, suelen ser más competitivos en la movilización del nitrógeno, fósforo y otros nutrientes (Smith y Read, 2008). El desarrollo de interacciones mutualistas planta-hongo micorrícico puede ser fundamental para el desarrollo de la cubierta vegetal en suelos muy degradados, donde la disponibilidad de nutrientes para la planta puede ser considerablemente baja.

Los hongos micorrícicos arbusculares son los hongos simbióticos más abundantes; colonizan de forma obligada a una gran variedad de hospedadores, dependiendo de la planta para obtener el carbono orgánico. Pertenecen únicamente a la división Glomeromycota, con hasta 1600 taxones descubiertos, pero se hospedan en unas 200000 especies de plantas diferentes (van der Heijden et al., 2015).

Los hongos ectomicorrícicos colonizan principalmente plantas leñosas, tanto angiospermas como gimnospermas. Estos organismos no son simbiontes obligados y tienen cierta capacidad saprófita. La simbiosis ectomicorrícica la pueden realizar una gran diversidad de hongos (unos 20000 taxones diferentes) de diferentes divisiones, perteneciendo principalmente a las divisiones Basidiomycota y Ascomycota (Smith y Read, 2008). Sin embargo, estos hongos interaccionan con un número mucho menor de plantas que los hongos micorrícicos arbusculares, en torno a unas 6000 especies de plantas (van der Heijden et al., 2015), y hay tanto especies hospedadoras específicas como generalistas (Taylor y Sinsabaugh, 2015).

Los hongos micorrícicos son reconocidos por su importancia en la absorción de nutrientes para la planta (Köhler et al., 2018; Tibbett y Sanders, 2002; van der Heijden et al., 2015). Chen et al. (2018) han sugerido recientemente que existe una co-evolución entre las plantas y los hongos, basada en la complementariedad funcional entre el hongo y la raíz en relación a la capacidad de buscar nutrientes.

En el estudio realizado por Phillips et al. (2013) mostraron como el papel ecológico de los hongos micorrícicos varía según el tipo de simbiosis micorrícica que se establece entre

el hongo y la planta. Descubrieron en bosques templados que en las zonas con dominancia de hongos micorrícicos arbusculares la calidad de la hojarasca y su descomposición era mayor que en las zonas con dominancia de hongos ectomicorrícicos. El horizonte orgánico aumentaba en estas últimas zonas mostrando una mayor relación C:N y mayor acidez, además de un mayor ciclado de nutrientes orgánicos (Fig. 1.4). Por lo tanto, en función de las especies de árboles introducidas durante un programa de fitorremediación y las interacciones que estas especies de árboles establezcan con las comunidades de hongos del suelo, la evolución a largo plazo del funcionamiento del suelo degradado sometido al proceso de restauración puede ser bastante diferente.

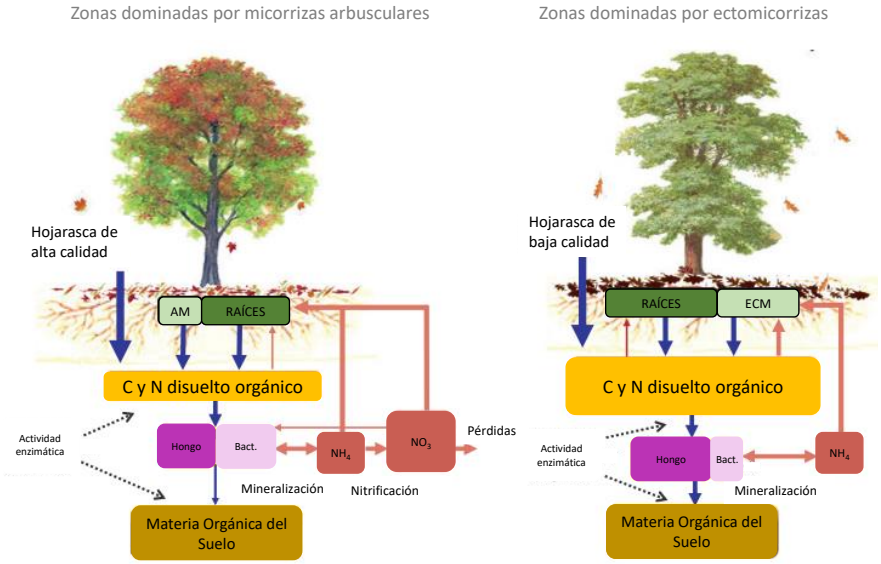


Figura 1.4 Esquema de las diferencias en el ciclado de nutrientes y carbono en bosques templados en zonas dominadas por micorrizas arbusculares o ectomicorrizas (traducido de Phillips et al. (2013)).

Uno de los procesos edáficos en los que el tipo de comunidad fúngica puede ejercer mayor influencia es el ciclado de carbono en el suelo. Los hongos ectomicorrícos favorecen la acumulación de carbono en los horizontes orgánicos, ya que reciben una mayor cantidad de carbono de la planta y al facilitar el transporte y la absorción de nitrógeno por ésta, se promueve un aumento de materia orgánica relativamente recalcitrante, con una alta relación C:N, formando un horizonte orgánico más grueso y más humificado (Clemmensen et al., 2013). Se estima que globalmente en los ecosistemas terrestres

dominados por ectomicorrizas se acumula 1.7 veces más carbono por unidad de nitrógeno que en los dominados por micorrizas arbusculares (Averill et al., 2014).

Parte de la influencia en estos procesos ecosistémicos está relacionada con los rasgos específicos del micelio (Koide et al., 2014a). El micelio de las ectomicorrizas genera una alta biomasa y tiene tasas de descomposición bajas, por lo que estos residuos pueden formar un 50-60 % del carbono secuestrado en el suelo, mientras que el micelio de las micorrizas arbusculares apenas llega al 5 % del carbono secuestrado en el suelo (Soudzilovskaia et al., 2015). La manera en la que los hongos ectomicorrícicos invierten en su estructura morfológica determina la capacidad exploratoria del micelio. Los tipos de exploración se distinguen por el desarrollo del micelio y de los rizomorfos, que son conjuntos de hifas especializadas que pueden transportar agua y nutrientes entre largas distancias (Agerer, 2006, 2001). Las especies de hongos ectomicorrícicos que producen rizomorfos crecen más rápidamente, producen más biomasa y son más longevas que las que tienen tipos de exploración cortos, contribuyendo así a una mayor inmovilización microbiana de carbono, nitrógeno y fósforo (Soudzilovskaia et al., 2015; Treseder y Lennon, 2015).

Existe otro rasgo en los hongos ectomicorrícicos con implicaciones en los procesos ecosistémicos; el contenido en melanina en las paredes celulares (Koide et al., 2014). Esta melanina tiene una naturaleza recalcitrante por lo que existe una relación inversamente proporcional con la tasa de descomposición del micelio (Fernandez y Koide, 2014). Por lo tanto, tiene el potencial de influir en la dinámica y secuestro del carbono en el suelo (Clemmensen et al., 2015). El contenido en melanina es considerado un rasgo morfológico con funciones de protección frente a múltiples factores de estrés abióticos como la salinidad, el estrés hídrico, la radiación ultravioleta, la contaminación o los metales pesados (Treseder y Lennon, 2015).

Por todos estos motivos, el estudio de las interacciones planta-ectomicorriza es muy relevante en ecosistemas emergentes, como los sometidos a programas de fitorremediación. Además de por la influencia en los procesos relacionados con la acumulación de carbono, la asociación simbiótica entre la raíz y el hongo micorrícico puede jugar un papel muy importante en el establecimiento de la planta en suelos degradados, dado que a través de esta simbiosis se puede mejorar la resistencia de la planta a diversos agentes estresantes como la sequía, la salinidad, los patógenos o los elementos traza, entre otros (van der Heijden et al., 2015). Por lo tanto, la mediación de

la micorriza en el estado de la planta podría verse potenciada en medios especialmente perturbados, como los suelos contaminados por elementos traza.

Numerosos estudios han mostrado que la simbiosis micorrícica juega un importante papel en la absorción de agua y nutrientes, y mejora la tolerancia de la planta a los elementos traza en suelos degradados, promoviendo la recuperación de las funciones en estos suelos (Cabral et al., 2015; Firmin et al., 2015; Smith y Read, 2008; van der Heijden y Scheublin, 2007). En suelos contaminados el establecimiento de las comunidades de plantas conlleva cambios en la estructura de la comunidad fúngica del suelo, y se potencian las asociaciones micorrícicas (Op De Beeck et al., 2015; van der Heijden et al., 1998). Por ello, las comunidades de hongos parecen estar más influenciadas por las comunidades de plantas que por las propiedades abióticas del suelo en casos de contaminación (Mueller et al., 2014). La producción primaria podría ser inhibida en suelos con concentraciones elevadas de metales, pero los efectos positivos y protectores de las micorrizas permiten el desarrollo vegetal (Krpata et al., 2009). La toxicidad de los elementos traza puede ser mitigada por la capacidad de translocar y secuestrar estos elementos en complejos químicos inaccesibles en los hongos (Taylor y Sinsabaugh, 2015). En el caso de los hongos ectomicorrícicos, la protección frente a metales se produce mediante mecanismos de evitación, como la precipitación extracelular, la biosorción en las paredes celulares o la reducción de la absorción, o mediante mecanismos de secuestro, como la quelación intracelular o la compartimentalización en vacuolas (Bellion et al., 2006; Hartley et al., 1997; Jentschke y Godbold, 2000). Por consiguiente, la recuperación mediante técnicas de fitorremediación en suelos contaminados por elementos traza puede beneficiarse de las interacciones planta-hongo en su objetivo de que las comunidades vegetales se puedan establecer y desarrollar con éxito (Wen et al., 2017).

1.9 Bibliografía

Abaye, D.A., Lawlor, K., Hirsch, P.R., Brookes, P.C., 2005. Changes in the microbial community of an arable soil caused by long-term metal contamination. European Journal of Soil Science 56, 93-102.

Agerer, R., 2006. Fungal relationships and structural identity of their ectomycorrhizae. Mycological Progress 5, 67-107.

Agerer, R., 2001. Exploration types of ectomycorrhizae: a proposal to classify ectomycorrhizal mycelial systems according to their patterns of differentiation and putative ecological importance. Mycorrhiza 11, 107-114.

Aksoy, E., Louwagie, G., Gardi, C., Gregor, M., Schröder, C., Löhnertz, M., 2017. Assessing soil biodiversity potentials in Europe. Science of the Total Environment 589, 236-249.

Ali, A., Guo, D., Mahar, A., Wang, P., Shen, F., Li, R., Zhang, Z., 2017. Mycoremediation of potentially toxic trace elements—a biological tool for soil cleanup: a review. Pedosphere 27, 205-222.

Ali, H., Khan, E., Sajad, M.A., 2013. Phytoremediation of heavy metals- Concepts and applications. Chemosphere 91, 869-881.

Angst, G., Mueller, K.E., Eissenstat, D.M., Trumbore, S., Freeman, K.H., Hobbie, S.E., Chorover, J., Oleksyn, J., Reich, P.B., Mueller, C.W., 2018. Soil organic carbon stability in forests: distinct effects of tree species identity and traits. Global Change Biology 25, 1-18.

Aponte, C., García, L. V., Marañón, T., 2013. Tree species effects on nutrient cycling and soil biota: a feedback mechanism favouring species coexistence. Forest Ecology and Management 309, 36-46.

Averill, C., Turner, B.L., Finzi, A.C., 2014. Mycorrhiza-mediated competition between plants and decomposers drives soil carbon storage. Nature 505, 543-545.

Bakker, P.A.H.M., Pieterse, C.M.J., de Jonge, R., Berendsen, R.L., 2018. The soil-borne legacy. Cell 172, 1178-1180.

Balestrini, R., Lumini, E., Borriello, R., Bianciotto, V., 2015. Plant-soil biota interactions, en: Paul, E.A. (Ed.), Soil Microbiology, Ecology and Biochemistry. Elsevier Inc., pp. 311-338.

Banning, N.C., Gleeson, D.B., Grigg, A.H., Grant, C.D., Andersen, G.L., Brodie, E.L., Murphy, D. V., 2011. Soil microbial community successional patterns during forest ecosystem restoration. Applied and Environmental Microbiology 77, 6158-6164.

Bauman, D., Raspé, O., Meerts, P., Degreef, J., Muledi, J.I., Drouet, T., 2016. Multiscale assemblage of an ectomycorrhizal fungal community: the influence of host functional traits and soil properties in a 10-ha miombo forest. FEMS Microbiology Ecology 92, 1-14.

Bellion, M., Courbot, M., Jacob, C., Blaudez, D., Chalot, M., 2006. Extracellular and cellular mechanisms sustaining metal tolerance in ectomycorrhizal fungi. FEMS Microbiology Letters 254, 173-181.

Berg, J., McClaugherty, C., 2014. Plant litter, 3rd ed. Springer-Verlag, Berlin, Heidelberg.

Bever, J.D., 2003. Soil community feedback and the coexistence of competitors: conceptual frameworks and empirical tests. New Phytologist 157, 465-473.

Bissett, A., Brown, M. V., Siciliano, S.D., Thrall, P.H., 2013. Microbial community responses to anthropogenically induced environmental change: towards a systems approach. Ecology Letters 16, 128-139.

Bolan, N.S., Park, J.H., Robinson, B., Naidu, R., Huh, K.Y., 2011. Phytostabilization: a green approach to contaminant containment. Advances in Agronomy 112, 145-204.

Brookes, P.C., 1995. The use of microbial parameters in monitoring soil pollution by heavy metals. Biology and Fertility of Soils 19, 269-279.

Bünemann, E.K., Bongiorno, G., Bai, Z., Creamer, R.E., De Deyn, G., de Goede, R., Fleskens, L., Geissen, V., Kuyper, T.W., Mäder, P., Pulleman, M., Sukkel, W., van Groenigen, J.W., Brussaard, L., 2018. Soil quality – A critical review. Soil Biology and Biochemistry 120, 105-125.

Cabral, L., Soares, C.R.F.S., Giachini, A.J., Siqueira, J.O., 2015. Arbuscular mycorrhizal fungi in phytoremediation of contaminated areas by trace elements: mechanisms and major benefits of their applications. World Journal of Microbiology and Biotechnology 31, 1655-1664.

Carnol, M., Bazgir, M., 2013. Nutrient return to the forest floor through litter and throughfall under 7 forest species after conversion from Norway spruce. Forest Ecology and Management 309, 66-75.

Chai, Y., Cao, Y., Yue, M., Tian, T., Yin, Q., Dang, H., Quan, J., Zhang, R., Wang, M., 2019. Soil abiotic properties and plant functional traits mediate associations between soil microbial and plant communities during a secondary forest succession on the Loess Plateau. Frontiers in Microbiology 10, 1-15.

Chander, K., Joergensen, R.G., 2001. Decomposition of 14C glucose in two soils with different amounts of heavy metal contamination. Soil Biology and Biochemistry 33, 1811-1816.

Chen, W., Eissenstat, D.M., Koide, R.T., 2018. Root diameter predicts the extramatrical hyphal exploration distance of the ectomycorrhizal fungal community. Ecosphere 9, 1-10.

Clarholm, M., Skyllberg, U., 2013. Translocation of metals by trees and fungi regulates pH, soil organic matter turnover and nitrogen availability in acidic forest soils. Soil Biology and Biochemistry 63, 142-153.

Clemente, R., Almela, C., Bernal, M.P., 2006. A remediation strategy based on active phytoremediation followed by natural attenuation in a soil contaminated by pyrite waste. Environmental Pollution 143, 397-406.

Clemmensen, K.E., Bahr, A., Ovaskainen, O., Dahlberg, A., Ekblad, A., Wallander, H., Stenlid, J., Finlay, R.D., Wardle, D.A., Lindahl, B.D., 2013. Roots and associated fungi drive long-term carbon sequestration in boreal forest. Science 339, 1615-1618.

Clemmensen, K.E., Finlay, R.D., Dahlberg, A., Stenlid, J., Wardle, D.A., Lindahl, B.D., 2015. Carbon sequestration is related to mycorrhizal fungal community shifts during long-term succession in boreal forests. New Phytologist 205, 1525-1536.

Comisión de las Comunidades Europeas, 2006. Comunicación de la Comisión al Consejo, al Parlamento Europeo, al Comité Económico y Social Europeo y al Comité de las Regiones. Estrategia temática para la protección del suelo, COM(2006)231.

Córdova, S.C., Olk, D.C., Dietzel, R.N., Mueller, K.E., Archontouilis, S. V., Castellano, M.J., 2018. Plant litter quality affects the accumulation rate, composition, and stability of mineral-associated soil organic matter. Soil Biology and Biochemistry 125, 115-124.

Cornelissen, J., Aerts, R., Cerabolini, B., Werger, M., Van der Heijden, M., 2001. Carbon cycling traits of plant species are linked with mycorrhizal strategy. Oecologia 129, 611-619.

Courty, P.E., Buée, M., Tech, J.J.T.T., Brulé, D., Colin, Y., Leveau, J.H.J.J., Uroz, S., 2018. Impact of soil pedogenesis on the diversity and composition of fungal communities across the California soil chronosequence of Mendocino. Mycorrhiza 28, 343-356.

Courty, P.E., Franc, A., Pierrat, J.C., Garbaye, J., 2008. Temporal changes in the ectomycorrhizal community in two soil horizons of a temperate oak forest. Applied and Environmental Microbiology 74, 5792-5801.

Cox, F., Barsoum, N., Lilleskov, E.A., Bidartondo, M.I., 2010. Nitrogen availability is a primary determinant of conifer mycorrhizas across complex environmental gradients. Ecology Letters 13, 1103-1113.

de Vries, F.T., Manning, P., Tallowin, J.R.B., Mortimer, S.R., Pilgrim, E.S., Harrison, K.A., Hobbs, P.J., Quirk, H., Shipley, B., Cornelissen, J.H.C., Kattge, J., Bardgett, R.D., 2012. Abiotic drivers and plant traits explain landscape-scale patterns in soil microbial communities. Ecology Letters 15, 1230-1239.

Domínguez, M.T., Marañón, T., Murillo, J.M., Schulin, R., Robinson, B.H., 2008. Trace element accumulation in woody plants of the Guadiamar Valley, SW Spain: a large-scale phytomanagement case study. Environmental Pollution 152, 50-59.

Erktan, A., McCormack, M.L., Roumet, C., 2018. Frontiers in root ecology: recent advances and future challenges. Plant and Soil 424, 1-9.

European Environment Agency, 2019. Land and soil in Europe: why we need to use these vital and finite resources sustainably, EEA SIGNALS 2019. Luxembourg.

European Environment Agency, United Nation Enviromental Programme, 2000. Down to Earth: soil degradation and sustainable development in Europe. Luxembourg.

Eviner, V.T., Hawkes, C. V., 2008. Embracing variability in the application of plant-soil interactions to the restoration of communities and ecosystems. Restoration Ecology 16, 713-729.

Fernandez, C.W., Koide, R.T., 2014. Initial melanin and nitrogen concentrations control the decomposition of ectomycorrhizal fungal litter. Soil Biology and Biochemistry 77, 150-157.

Fierer, N., 2017. Embracing the unknown: Disentangling the complexities of the soil microbiome. Nature Reviews Microbiology 15, 579-590.

Fierer, N., Strickland, M.S., Liptzin, D., Bradford, M.A., Cleveland, C.C., 2009. Global patterns in belowground communities. Ecology Letters 12, 1238-1249.

Firmin, S., Labidi, S., Fontaine, J., Laruelle, F., Tisserant, B., Nsanganwimana, F., Pourrut, B., Dalpé, Y., Grandmougin, A., Douay, F., Shirali, P., Verdin, A., Lounès-Hadj Sahraoui, A., 2015. Arbuscular mycorrhizal fungal inoculation protects Miscanthus × giganteus against trace element toxicity in a highly metal-contaminated site. Science of the Total Environment 527-528, 91-99.

Friesen, M.L., Porter, S.S., Stark, S.C., von Wettberg, E.J., Sachs, J.L., Martinez-Romero, E., 2011. Microbially Mediated Plant Functional Traits. Annual Review of Ecology, Evolution, and Systematics 42, 23–46.

Garbisu, C., Hernández-Allica, J., Barrutia, O., Alkorta, I., Becerril, J.M., 2002. Phytoremediation: a technology using green plants to remove contaminants from polluted areas. Reviews on Environmental Health 17, 173-188.

Giller, K.E., Witter, E., Mcgrath, S.P., 1998. Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. Soil Biology and Biochemistry 30, 1389-1414.

Griffiths, B.S., Philippot, L., 2012. Insights into the resistance and resilience of the soil microbial community. FEMS Microbiology Reviews 37, 112-129.

Grosso, F., Iovieno, P., Alfani, A., Nicola, F. De, 2018. Structure and activity of soil microbial communities in three Mediterranean forests. Applied Soil Ecology 130, 280-287.

Harris, J., 2009. Soil microbial communities and restoration ecology: facilitators or followers? Science 325, 573-574.

Harris, J.A., 2003. Measurements of the soil microbial community for estimating the success of restoration. European Journal of Soil Science 54, 801-808.

Hartley, J., Cairney, J.W.G., Meharg, A.A., 1997. Do ectomycorrhizal fungi exhibit adaptive tolerance to potentially toxic metals in the environment? Plant and Soil 189, 303-319.

Hooda, P.S., 2010. Trace elements in soils, 1st ed. Blackwell Publishing.

Jentschke, G., Godbold, D.L., 2000. Metal toxicity and ectomycorrhizas. Physiologia Plantarum 109, 107-116.

Joergensen, R.G., Wichern, F., 2008. Quantitative assessment of the fungal contribution to microbial tissue in soil. Soil Biology and Biochemistry 40, 2977-2991.

Kabata-Pendias, A., Pendias, H., 2001. Trace elements in soils and plants, 3rd ed. CRC Press LLC, Boca Raton.

Kandeler, E., Tscherko, D., Bruce, K.D., Stemmer, M., Hobbs, P.J., Bardgett, R.D., Amelung, W., 2000. Structure and function of the soil microbial community in microhabitats of a heavy metal polluted soil. Biology and Fertility of Soils 32, 390-400.

Kardol, P., Wardle, D.A., 2010. How understanding aboveground-belowground linkages can assist restoration ecology. Trends in Ecology and Evolution 25, 670-679.

Kennedy, P., 2010. Ectomycorrhizal fungi and interspecific competition: species interactions, community structure, coexistence mechanisms, and future research directions. New Phytologist 187, 895-910.

Kennedy, P.G., Peay, K.G., Bruns, T.D., 2009. Root tip competition among ectomycorrhizal fungi: are priority effects a rule or an exception? Ecology 90, 2098-2107.

Khan, A.G., 2005. Role of soil microbes in the rhizospheres of plants growing on trace metal contaminated soils in phytoremediation. Journal of Trace Elements in Medicine and Biology 18, 355-364.

Khan, S., Hesham, A.E.L., Qiao, M., Rehman, S., He, J.Z., 2010. Effects of Cd and Pb on soil microbial community structure and activities. Environmental Science and Pollution Research 17, 288-296.

Kögel-Knabner, I., 2002. The macromolecular organic composition of plant and microbial residues as inputs to soil organic matter. Soil Biology and Biochemistry 34, 139-162.

Köhler, J., Yang, N., Pena, R., Raghavan, V., Polle, A., Meier, I.C., 2018. Ectomycorrhizal fungal diversity increases phosphorus uptake efficiency of European beech. New Phytologist 220, 1200-1210.

Koide, R.T., Fernandez, C., Malcolm, G., 2014. Determining place and process: functional traits of ectomycorrhizal fungi that affect both community structure and ecosystem function. New Phytologist 201, 433-439.

Koide, R.T., Sharda, J.N., Herr, J.R., Malcolm, G.M., 2008. Ectomycorrhizal fungi and the biotrophy–saprotrophy continuum. New Phytologist 178, 230-233.

Krpata, D., Fitz, W., Peintner, U., Langer, I., Schweiger, P., 2009. Bioconcentration of zinc and cadmium in ectomycorrhizal fungi and associated aspen trees as affected by level of pollution. Environmental Pollution 157, 280-286.

Krumins, J.A., Goodey, N.M., Gallagher, F., 2015. Plant-soil interactions in metal contaminated soils. Soil Biology and Biochemistry 80, 224-231.

Kuiper, I., Lagendijk, E.L., Bloemberg, G. V, Lugtenberg, B.J.J., 2004. Rhizoremediation: a beneficial plant-microbe interaction. Molecular Plant-Microbe Interactions 17, 6-15.

Kumpiene, J., Antelo, J., Brännvall, E., Carabante, I., Ek, K., Komárek, M., Söderberg, C., Wårell, L., 2019. In situ chemical stabilization of trace element-contaminated soil – Field demonstrations and barriers to transition from laboratory to the field – A review. Applied Geochemistry 100, 335-351.

Landeweert, R., Leeflang, P., Kuyper, T.W., Hoffland, E., Rosling, A., Wernars, K., Smit, E., 2003. Molecular identification of ectomycorrhizal mycelium in soil horizons. Applied and Environmental Microbiology 69, 327-333.

Langley, J.A., Hungate, B.A., 2003. Mycorrhizal controls on belowground litter quality. Ecology 84, 2302-2312.

Lindahl, B.D., Ihrmark, K., Boberg, J., Trumbore, S.E., Högberg, P., Stenlid, J., Finlay, R.D., 2007. Spatial separation of litter decomposition and mycorrhizal nitrogen uptake in a boreal forest. New Phytologist 173, 611-620.

López-García, Á., Varela-Cervero, S., Vasar, M., Öpik, M., Barea, J.M., Azcón-Aguilar, C., 2017. Plant traits determine the phylogenetic structure of arbuscular mycorrhizal fungal communities. Molecular Ecology 26, 6948-6959.

Madejón, P., Domínguez, M.T., Gil-Martínez, M., Navarro-Fernández, C.M., Montiel-Rozas, M.M., Madejón, E., Murillo, J.M., Cabrera, F., Marañón, T., 2018a. Evaluation of amendment addition and tree planting as measures to remediate contaminated soils: the Guadiamar case study (SW Spain). Catena 166, 34-43.

Madejón, P., Domínguez, M.T., Madejón, E., Cabrera, F., Marañón, T., Murillo, J.M., 2018b. Soil-plant relationships and contamination by trace elements: a review of twenty years of experimentation and monitoring after the Aznalcóllar (SW Spain) mine accident. Science of the Total Environment 625, 50-63.

Madejón, P., Gil-Martínez, M., Marañón, T., 2020. Fitorrecuperación de suelos contaminados en el Corredor Verde del Guadiamar, en: Madejón, P., Marañón, T. (Eds.), Recuperación de suelos y servicios ecosistémicos en el Corredor Verde del Guadiamar. Editorial CSIC, Madrid, pp. 93-112.

Maghnia, F.Z., Abbas, Y., Mahé, F., Kerdouh, B., Tournier, E., Ouadji, M., Tisseyre, P., Prin, Y., Ghachtouli, N. El, Bakkali Yakhlef, S.E., Duponnois, R., Sanguin, H., 2017. Habitat- and soil-related drivers of the root-associated fungal community of Quercus suber in the Northern Moroccan forest. PLoS ONE 12, e0187758.

Marañón, T., Navarro-Fernández, C.M., Domínguez, M.T., Madejón, P., Murillo, J.M., 2015. How the soil chemical composition is affected by seven tree species planted at a contaminated and remediated site. Web Ecology 15, 45-48.

Mendez, M.O., Maier, R.M., 2008. Phytostabilization of mine tailings in arid and semiarid environments - An emerging remediation technology. Environmental Health Perspectives 116, 278-283.

Mitchell, R.J., Campbell, C.D., Chapman, S.J., Cameron, C.M., 2010. The ecological engineering impact of a single tree species on the soil microbial community. Journal of Ecology 98, 50-61.

Moll, J., Hoppe, B., König, S., Wubet, T., Buscot, F., Krüger, D., 2016. Spatial distribution of fungal communities in an arable soil. PLoS ONE 11, e0148130.

Mueller, R.C., Paula, F.S., Mirza, B.S., Rodrigues, J.L.M., Nüsslein, K., Bohannan, B.J.M., 2014. Links between plant and fungal communities across a deforestation chronosequence in the Amazon rainforest. ISME Journal 8, 1548-1550.

Muñoz-Rojas, M., 2018. Soil quality indicators: critical tools in ecosystem restoration. Current Opinion in Environmental Science and Health 5, 47-52.

Nannipieri, P., Ascher, J., Ceccherini, M.T., Landi, L., Pietramellara, G., Renella, G., 2017. Microbial diversity and soil functions. European Journal of Soil Science 68, 12-26.

Naranjo-Ortiz, M.A., Gabaldón, T., 2019. Fungal evolution: diversity, taxonomy and phylogeny of the Fungi. Biological Reviews 94, 2101-2137.

Narendrula-Kotha, R., Nkongolo, K.K., 2017. Bacterial and fungal community structure and diversity in a mining region under long-term metal exposure revealed by metagenomics sequencing. Ecological Genetics and Genomics 2, 13-24.

Nilsson, R.H., Anslan, S., Bahram, M., Wurzbacher, C., Baldrian, P., Tedersoo, L., 2019. Mycobiome diversity: high-throughput sequencing and identification of fungi. Nature Reviews Microbiology 17, 95-109.

Op De Beeck, M., Lievens, B., Busschaert, P., Rineau, F., Smits, M., Vangronsveld, J., Colpaert, J. V., 2015. Impact of metal pollution on fungal diversity and community structures. Environmental Microbiology 17, 2035-2047.

Orgiazzi, A., Dunbar, M.B., Panagos, P., de Groot, G.A., Lemanceau, P., 2015. Soil biodiversity and DNA barcodes: opportunities and challenges. Soil Biology and Biochemistry 80, 244-250.

Orozco-Aceves, M., Standish, R.J., Tibbett, M., 2015. Long-term conditioning of soil by plantation eucalypts and pines does not affect growth of the native jarrah tree. Forest Ecology and Management 338, 92-99.

Panagos, P., Van Liedekerke, M., Yigini, Y., Montanarella, L., 2013. Contaminated sites in Europe: review of the current situation based on data collected through a European network. Journal of Environmental and Public Health 2013, 1-11.

Peršoh, D., 2015. Plant-associated fungal communities in the light of meta'omics. Fungal Diversity 75, 1-25.

Phillips, R.P., Brzostek, E., Midgley, M.G., 2013. The mycorrhizal-associated nutrient economy: a new framework for predicting carbon-nutrient couplings in temperate forests. New Phytologist 199, 41-51.

Prescott, C.E., Grayston, S.J., 2013. Tree species influence on microbial communities in litter and soil: current knowledge and research needs. Forest Ecology and Management 309, 19-27.

Pulford, I.D., Watson, C., 2003. Phytoremediation of heavy metal-contaminated land by trees - A review. Environment International 29, 529-540.

Quilchano, C., Marañón, T., 2002. Dehydrogenase activity in Mediterranean forest soils. Biology and Fertility of Soils 35, 102-107.

Richards, A.E., Forrester, D.I., Bauhus, J., Scherer-Lorenzen, M., 2010. The influence of mixed tree plantations on the nutrition of individual species: a review. Tree Physiology 30, 1192-1208.

Robinson, B.H., Bañuelos, G., Conesa, H.M., Evangelou, M.W.H., Schulin, R., 2009. The phytomanagement of trace elements in soil. Critical Reviews in Plant Sciences 28, 240-266.

Robinson, B.H., Fernandez, J.E., Madejon, P., Matarien, T., Murillo, J.M., Green, S., Clothier, B., 2003. Phytoextraction: an assessment of biochemical and economic viability. Plant and Soil 249, 117-125.

Rodríguez-Eugenio, N., McLaughlin, M., Pennock, D., 2018. Soil pollution: a hidden reality. FAO, Rome.

Rousk, J., Brookes, P.C., Bååth, E., 2009. Contrasting soil pH effects on fungal and bacterial growth suggest functional redundancy in carbon mineralization. Applied and Environmental Microbiology 75, 1589-1596.

Rutten, G., Gómez-Aparicio, L., 2018. Plant-soil feedbacks and root responses of two Mediterranean oaks along a precipitation gradient. Plant and Soil 424, 221-231.

Schappe, T., Albornoz, F.E., Turner, B.L., Neat, A., Condit, R., Jones, F.A., 2017. The role of soil chemistry and plant neighbourhoods in structuring fungal communities in three Panamanian rainforests. Journal of Ecology 105, 569-579.

Schimel, J., Balser, T., Wallenstein, M.D., 2007. Microbial stress-response physiology and its implications for ecosystem function. Ecology 88, 1386-1394.

Schneider, T., Keiblinger, K.M., Schmid, E., Sterflinger-Gleixner, K., Ellersdorfer, G., Roschitzki, B., Richter, A., Eberl, L., Zechmeister-Boltenstern, S., Riedel, K., 2012. Who is who in litter decomposition? Metaproteomics reveals major microbial players and their biogeochemical functions. ISME Journal 6, 1749-1762.

Smith, S., Read, D., 2008. Mycorrhizal Symbiosis, 3rd ed. Academic Press, London.

Soudzilovskaia, N.A., van der Heijden, M.G.A., Cornelissen, J.H.C., Makarov, M.I., Onipchenko, V.G., Maslov, M.N., Akhmetzhanova, A.A., van Bodegom, P.M., 2015. Quantitative assessment of the differential impacts of arbuscular and ectomycorrhiza on soil carbon cycling. New Phytologist 208, 280-293.

Sun, H., Terhonen, E., Kovalchuk, A., Tuovila, H., Chen, H., Oghenekaro, A.O., Heinonsalo, J., Kohler, A., Kasanen, R., Vasander, H., Asiegbu, F.O., 2016. Dominant tree species and soil type affect the fungal community structure in a boreal peatland forest. Applied and Environmental Microbiology 82, 2632-2643.

Sun, S., Li, S., Avera, B.N., Strahm, B.D., Badgley, B.D., 2017. Soil bacterial and fungal communities show distinct recovery patterns during forest ecosystem restoration. Applied and Environmental Microbiology 83, 1-14.

Taylor, D.L., Herriott, I.C., Stone, K.E., Mcfarland, J.W., Booth, M.G., Leigh, M.B., 2010. Structure and resilience of fungal communities in alaskan boreal forest soils1. Canadian Journal of Forest Research 40, 1288-1301.

Taylor, D.L., Sinsabaugh, R.L., 2015. The soil fungi: ocurrence, phylogeny, and ecology, en: Paul, E.A. (Ed.), Soil Microbiology, Ecology and Biochemistry. Elsevier Inc., pp. 77-109.

Tedersoo, L., Bahram, M., Põlme, S., Kõljalg, U., Yorou, N.S., Wijesundera, R., Ruiz, L.V., Vasco-Palacios, A.M., Thu, P.Q., Suija, A., Smith, M.E., Sharp, C., Saluveer, E., Saitta, A., Rosas, M., Riit, T., Ratkowsky, D., Pritsch, K., Põldmaa, K., Piepenbring, M., Phosri, C., Peterson, M., Parts, K., Pärtel, K., Otsing, E., Nouhra, E., Njouonkou, A.L., Nilsson, R.H., Morgado, L.N., Mayor, J., May, T.W., Majukim, L., Lodge, D.J., Lee, S.S., Larsson, K.-H., Kohout, P., Hosaka, K., Hiiesalu, I., Henkel, T.W., Harend, H., Guo, L., Greslebin, A., Gretlet, G., Geml, J., Gates, G., Dunstan, W., Dunk, C., Drenkhan, R., Dearnaley, J., Kesel, A. De, Dang, T., Chen, X., Buegger, F., Brearley, F.Q., Bonito, G., Anslan, S., Abell, S., Abarenkov, K., 2014. Global diversity and geography of soil fungi. Science 346, 1256688.

Tedersoo, L., Bahram, M., Toots, M., Diédhiou, A.G., Henkel, T.W., Kjoller, R., Morris, M.H., Nara, K., Nouhra, E., Peay, K.G., Põlme, S., Ryberg, M., Smith, M.E., Kõljalg, U., 2012. Towards global patterns in the diversity and community structure of ectomycorrhizal fungi. Molecular Ecology 21, 4160-4170.

Tedersoo, L., Koljalg, U., Hallenberg, N., Larsson, K.-H., 2003. Fine scale distribution of ectomycorrhizal fungi and roots across substrate layers including coarse woody debris in a mixed forest. New Phytologist 159, 153-165.

Thorn, R.G., Lynch, M.D.J., 2006. Fungi and eukaryotic algae, en: Paul, E.A. (Ed.), Soil Microbiology, Ecology and Biochemistry. Elsevier Inc., pp. 145-162.

Tibbett, M., Sanders, F.E., 2002. Ectomycorrhizal symbiosis can enhance plant nutrition through improved access to discrete organic nutrient patches of high resource quality. Annals of Botany 89, 783-789.

Treseder, K.K., Lennon, J.T., 2015. Fungal traits that drive ecosystem dynamics on land. Microbiology and Molecular Biology Reviews 79, 243-262.

Unterseher, M., Schnittler, M., Dormann, C., Sickert, A., 2008. Application of species richness estimators for the assessment of fungal diversity. FEMS Microbiology Letters 282, 205-213.

Uroz, S., Courty, P.E., Oger, P., 2019. Plant symbionts are engineers of the plant-associated microbiome. Trends in Plant Science 24, 905-916.

van der Bij, A.U., Weijters, M.J., Bobbink, R., Harris, J.A., Pawlett, M., Ritz, K., Benetková, P., Moradi, J., Frouz, J., van Diggelen, R., 2018. Facilitating ecosystem assembly: plant-soil interactions as a restoration tool. Biological Conservation 220, 272-279.

van der Heijden, M.G.A., Bardgett, R.D., Van Straalen, N.M., 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecology Letters 11, 296-310.

van der Heijden, M.G.A., Klironomos, J.N., Ursic, M., Moutoglis, P., Streitwolf-Engel, R., Boller, T., Wiemken, A., Sanders, I.R., 1998. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature 396, 69-72.

van der Heijden, M.G.A., Martin, F.M., Selosse, M.-A., Sanders, I.R., 2015. Mycorrhizal ecology and evolution: the past, the present, and the future. New Phytologist 205, 1406-1423.

van der Heijden, M.G.A., Scheublin, T.R., 2007. Functional traits in mycorrhizal ecology: their use for predicting the impact of arbuscular mycorrhizal fungal communities on plant growth and ecosystem functioning. New Phytologist 174, 244-250.

van der Putten, W.H., Bardgett, R.D., Bever, J.D., Bezemer, T.M., Casper, B.B., Fukami, T., Kardol, P., Klironomos, J.N., Kulmatiski, A., Schweitzer, J.A., Suding, K.N., Van de Voorde, T.F.J., Wardle, D.A., 2013. Plant-soil feedbacks: the past, the present and future challenges. Journal of Ecology 101, 265-276.

van der Wal, A., van Veen, J.A., Smant, W., Boschker, H.T.S., Bloem, J., Kardol, P., van der Putten, W.H., De Boer, W., 2006. Fungal biomass development in a chronosequence of land abandonment. Soil Biology and Biochemistry 38, 51-60.

van Liedekerke, M., Prokop, G., Rabl-Berger, S., Kibblewhite, M., Louwagie, G., 2014. Progress in the management of contaminated sites in Europe. Luxembourg.

Vasco-Palacios, A.M., Bahram, M., Boekhout, T., Tedersoo, L., 2020. Carbon content and pH as important drivers of fungal community structure in three Amazon forests. Plant and Soil 450, 111–131.

Voříšková, J., Baldrian, P., 2013. Fungal community on decomposing leaf litter undergoes rapid successional changes. ISME Journal 7, 477-486.

Waldrop, M.P., Holloway, J.M., Smith, D.B., Goldhaber, M.B., Drenovsky, R.E., Scow, K.M., Dick, R., Howard, D., Wylie, B., Grace, J.B., 2017. The interacting roles of climate, soils, and plant production on soil microbial communities at a continental scale. Ecology 98, 1957-1967.

Wang, N., Wang, A., Xie, J., He, M., 2019. Responses of soil fungal and archaeal communities to environmental factors in an ongoing antimony mine area. Science of the Total Environment 652, 1030-1039.

Wardle, D.A., Bardgett, R.D., Klironomos, J.N., Setälä, H., Van Der Putten, W.H., Wall, D.H., 2004. Ecological linkages between aboveground and belowground biota. Science 304, 1629-1633.

Wen, Z., Shi, L., Tang, Y., Shen, Z., Xia, Y., Chen, Y., 2017. Effects of Pisolithus tinctorius and Cenococcum geophilum inoculation on pine in copper-contaminated soil to enhance phytoremediation. International Journal of Phytoremediation 19, 387-394.

Wilson, G.W.T., Rice, C.W., Rillig, M.C., Springer, A., Hartnett, D.C., 2009. Soil aggregation and carbon sequestration are tightly correlated with the abundance of arbuscular mycorrhizal fungi: results from long-term field experiments. Ecology Letters 12, 452-461.

Wójcik, M., Sugier, P., Siebielec, G., 2014. Metal accumulation strategies in plants spontaneously inhabiting Zn-Pb waste deposits. Science of the Total Environment 487, 313-322.

Zhou, L., Li, Z., Liu, W., Liu, S., Zhang, L., Zhong, L., Luo, X., Liang, H., 2015. Restoration of rare earth mine areas: organic amendments and phytoremediation. Environmental Science and Pollution Research 22, 17151-17160.

2. OBJETIVOS Y ESTRUCTURA DE LA TESIS

El objetivo general de esta tesis doctoral es investigar las interacciones planta-suelo-microorganismo en zonas contaminadas por elementos traza y reforestadas con distintas especies leñosas. Se estudian las comunidades microbianas del suelo, sus actividades y sus relaciones con los árboles en una zona degradada donde se implementó un programa de reforestación durante el año 1999. El estudio se ha realizado en el Corredor Verde del Guadiamar (Sevilla) (ver descripción en el **Capítulo 3**), que fue afectado por un vertido minero y recuperado aplicando una estrategia de fitorremediación.

La hipótesis de trabajo es que los procesos asociados a la fitorremediación se intensifican con el tiempo. Después de la plantación de diferentes especies de árboles y arbustos, y transcurridos algunos años de crecimiento, debe esperarse que los árboles empiecen a producir cambios en los suelos y la rizosfera, afectando a las comunidades microbianas que habitan en él, las cuales también estarán determinadas por las condiciones abióticas del suelo, especialmente por la contaminación residual de elementos traza. Al mismo tiempo, también deben esperarse efectos de los microorganismos del suelo sobre la nutrición y acumulación de elementos traza por las plantas, mediante procesos de retroalimentación (*feedback*).

El papel de la microbiota del suelo es crucial para el funcionamiento del ecosistema. El conjunto de estudios recogidos en esta tesis doctoral busca mejorar el conocimiento de la microbiota del suelo y sus funciones en este tipo de zonas degradadas. Desde el punto de vista aplicado, es importante conocer los efectos a medio plazo de las estrategias de fitorremediación de suelos contaminados y la recuperación de servicios ecosistémicos; en particular mediante la recuperación de las funciones y procesos que tienen lugar en el suelo. Los resultados obtenidos en esta tesis sobre la evaluación de los efectos remediadores de diferentes especies leñosas pueden ayudar a la gestión y restauración de otras zonas contaminadas por elementos traza en ecosistemas mediterráneos.

A continuación, se detallan los objetivos específicos y las hipótesis planteadas en los diferentes capítulos de la tesis.

En los **Capítulos 4 y 5** se estudia la influencia de diferentes especies leñosas y de diferentes niveles de contaminación de elementos traza en la composición y funcionamiento de la microbiota del suelo. Las especies forestales de estudio son el acebuche (*Olea europaea* var. *sylvestris*), el álamo blanco (*Populus alba*) y el pino piñonero (*Pinus pinea*). Se han elegido dos zonas de estudio dentro del Corredor Verde

del Guadiamar - zona Norte y zona Sur - que presentan de manera general valores contrastados en las concentraciones de elementos traza, así como en los niveles de pH y nutrientes.

En el **Capítulo 4** se estudia el funcionamiento del conjunto de comunidades microbianas que se desarrollan en estos suelos, a través del análisis del carbono y nitrógeno microbiano (biomasa), de algunas actividades enzimáticas producidas por los microorganismos implicados en los ciclos del carbono, nitrógeno y fósforo, y de la diversidad catabólica de la microbiota. Se comparan dos muestreos realizados en primavera y otoño para conocer el efecto de la estacionalidad (temperatura y humedad) en las funciones microbianas. Las cuestiones que se plantean en este capítulo son:

1) ¿De qué manera la contaminación por elementos traza del suelo produce una reducción de la biomasa y las actividades microbianas?

2) ¿En qué medida la forestación con diferentes especies leñosas induce cambios en las comunidades microbianas del suelo y en sus actividades?

3) ¿Cuál es la influencia de las variables abióticas en las interacciones de las especies leñosas con la microbiota del suelo?

4) ¿En qué medida los cambios estacionales (temperatura y humedad) son responsables de variaciones en las actividades microbianas del suelo?

En el **Capítulo 5** se estudia de manera específica la comunidad de hongos del suelo, un grupo de la biota edáfica con importantes funciones en el suelo, y que interacciona con las raíces de los árboles a través de relaciones simbióticas micorrícicas. En este capítulo se han utilizado técnicas de secuenciación masiva para conocer la abundancia relativa, la taxonomía y los gremios funcionales de las especies fúngicas del suelo. Se han estudiado los efectos de las especies de árboles en los índices de diversidad, en la funcionalidad y en la composición de las comunidades fúngicas del suelo subyacente. Las cuestiones que se plantean en este capítulo son:

1) ¿Cómo las medidas de fitorremediación, y especialmente la forestación, han favorecido una mayor diversidad de hongos en el suelo?

2) ¿En qué medida las comunidades fúngicas del suelo están afectadas por las variables bióticas (la cobertura vegetal y el tipo de especie arbórea) y por las variables abióticas (contaminación, pH y nutrientes)?

3) ¿Cómo las especies leñosas, a través de sus diferentes tipos de simbiosis, afectan a la diversidad funcional y de gremios de los hongos del suelo?

En los **Capítulos 6 y 7** se estudia el efecto de la contaminación por elementos traza en un grupo específico de la comunidad fúngica del suelo, las comunidades de hongos ectomicorrícicos que están en simbiosis con las raíces de la encina (*Quercus ilex* subsp. *ballota*), una especie leñosa muy abundante en el Corredor Verde del Guadiamar. Se han estudiado las interacciones ectomicorriza-encina en zonas contaminadas y no contaminadas de la zona Norte y zona Sur del Corredor Verde, con diferentes condiciones ambientales.

En el **Capítulo 6** se estudia el efecto de la contaminación por elementos traza en la composición, taxonomía y diversidad funcional de hongos ectomicorrícicos. Se aplican técnicas de secuenciación de Sanger y análisis de rasgos morfológicos de estos hongos (hifas, rizomorfos y melanina). Las cuestiones que se plantean en este capítulo son:

1) ¿Cuál es el efecto de la contaminación por elementos traza en la diversidad taxonómica de los hongos ectomicorrícicos asociados a la encina? ¿Cómo varía la composición taxonómica de las comunidades fúngicas entre suelos contaminados y suelos no contaminados?

2) ¿Cuál es el efecto de la contaminación en los rasgos morfológicos medios de la comunidad de hongos ectomicorrícicos? En particular, ¿cambia el tipo de exploración o el grado de melanización?

3) ¿De qué manera la contaminación del suelo actúa como un filtro ambiental reduciendo la diversidad de rasgos morfológicos de la comunidad de hongos ectomicorrícicos?

En el **Capítulo 7** se estudia la mediación de los hongos ectomicorrícicos en el estado nutricional de la encina. La interacción planta-suelo está afectada por factores abióticos y bióticos; aquí se estudia cómo los hongos ectomicorrícicos (su composición taxonómica

y sus rasgos morfológicos) pueden determinar ciertos rasgos de la encina (rasgos morfológicos de sus raíces y sus hojas), así como la transferencia de elementos traza del suelo a la planta. Las cuestiones que se plantean en este capítulo son:

1) ¿Cómo varían los rasgos morfológicos y químicos de las raíces y hojas de encina en un gradiente de contaminación?

2) ¿Cuál es el papel de mediación de los hongos ectomicorrícicos en la respuesta de la encina a la contaminación del suelo? Específicamente, ¿cómo pueden la composición de especies micorrícicas y los rasgos funcionales de estas especies explicar el estado nutritivo y la transferencia de elementos traza desde el suelo a las raíces y a las hojas de la encina?

3) ¿De qué manera las comunidades de hongos ectomicorrícicos lideran la variación intraespecífica de los rasgos funcionales en las raíces de la encina?

En el **Capítulo 8** se estudian dos especies de hongos comestibles (*Laccaria laccata*, hongo ectomicorrícico, y *Volvopluteus gloiocephalus*, hongo saprófito) que crecen naturalmente en los suelos contaminados de la zona Norte del Corredor Verde del Guadiamar. Se han analizado los nutrientes y el marcaje isotópico ($\delta^{13}C$, $\delta^{15}N$) en los cuerpos fructíferos (setas) y el suelo subyacente para conocer las diferentes dinámicas de las relaciones suelo-hongo según su tipo de nutrición (saprófito o mutualista). También, se han analizado las concentraciones de elementos traza para determinar la transferencia de tales elementos desde el suelo a la seta y evaluar el riesgo potencial para la salud humana derivado de su consumo. Las cuestiones que se plantean en este capítulo son:

1) ¿Qué diferencias existen en el tipo de nutrición entre ambas especies de hongos? ¿Cómo refleja la composición isotópica de los cuerpos fructíferos las diferencias en la obtención de carbono y nitrógeno entre las dos especies?

2) ¿Cuál es la capacidad de acumulación de elementos traza por las dos especies de setas? ¿Cómo se relacionan los patrones de acumulación en las setas con las concentraciones del suelo?

3) ¿Existe un riesgo toxicológico potencial por consumo humano de estas setas recolectadas en una zona contaminada con elementos traza?

2.1 Publicaciones derivadas de la tesis doctoral

Los diferentes capítulos de la tesis han dado lugar a diversas publicaciones científicas. A continuación, se detalla la información bibliográfica de cada una de ellas.

Capítulo 4

Gil-Martínez, Marta; Domínguez, María T.; Navarro-Fernández, Carmen M.; Tibbett, Mark y Marañón, Teodoro (2020). Soil microbial communities and their functionality are driven by soil properties, afforested tree species and drought stress in a Mediterranean trace element contaminated area (en preparación).

Gil-Martínez, Marta; Domínguez, María T.; Navarro-Fernández, Carmen M.; Crompot, Héloïse; Tibbett, Mark y Marañón, Teodoro (2018). Long-term effects of trace elements contamination on soil microbial biomass and enzyme activities, en C Drebenstedt, F von Bismarck, A Fourie & M Tibbett (eds), *Proceedings of the 12th International Conference on Mine Closure*, Technical University Bergakademie Freiberg, Germany, pp. 633-644. (Una versión preliminar con parte de los resultados fue publicada en las actas del simposio 12th International Conference on Mine Closure).

Capítulo 5

Gil-Martínez, Marta; López-García, Álvaro; Domínguez, María T.; Kjøller, Rasmus; Navarro-Fernández, Carmen M.; Rosendahl, Søren y Marañón, Teodoro (2020). Soil fungal diversity and functionality are driven by plant species used in phytoremediation (en revisión en la revista *Soil Biology and Biochemistry*).

Capítulo 6

López-García, Álvaro; Gil-Martínez, Marta; Navarro-Fernández, Carmen M.; Kjøller, Rasmus; Azcón-Aguilar, Concepción; Domínguez María T. y Marañón, Teodoro (2018). Functional diversity of ectomycorrhizal fungal communities is reduced by trace element contamination. *Soil Biology and Biochemistry 121: 202-211*.

Capítulo 7

Gil-Martínez, Marta; López-García, Álvaro; Domínguez, María T.; Navarro-Fernández, Carmen M.; Kjøller, Rasmus; Tibbett, Mark y Marañón, Teodoro (2018). Ectomycorrhizal fungal communities and their functional traits mediate plant–soil interactions in trace element contaminated soils. *Frontiers in Plant Science, 9: 1682*.

Capítulo 8

Gil-Martínez, Marta; Navarro-Fernández, Carmen M.; Murillo, José M.; Domínguez, María T. y Marañón, Teodoro (2020). Trace elements and C and N isotope composition in two mushroom species from a mine-spill contaminated site. *Scientific Reports, 10: 6434.*

2.2 Colaboración en otras publicaciones

Madejón, Paula; Domínguez, María T.; Gil-Martínez, Marta; Navarro-Fernández, Carmen M.; Montiel-Rozas, María del Mar; Madejón, Engracia; Murillo, José M.; Cabrera, Francisco y Marañón, Teodoro (2018). Evaluation of amendment addition and tree planting as measures to remediate contaminated soils: the Guadiamar case study (SW Spain). *Catena 166: 34- 43*.

Madejón, Paula; Gil-Martínez, Marta y Marañón, Teodoro (2020). Fitorrecuperación de suelos contaminados en el Corredor Verde del Guadiamar, en Madejón, Paula & Marañón, Teodoro (eds.), Recuperación de suelos y servicios ecosistémicos en el Corredor Verde del Guadiamar, Editorial CSIC, Madrid, España, pp. 93-112.

Marañón, Teodoro; Navarro-Fernández, Carmen M.; Gil-Martínez, Marta; Domínguez, María T.; Madejón, Paula y Villar, Rafael (2020). Variation in morphological and chemical traits of Mediterranean tree roots: linkage with leaf traits and soil conditions. *Plant Soil 449: 389–403*.

3. ÁREA DE ESTUDIO

Los estudios de las interacciones planta-suelo-microorganismo en zonas contaminadas por elementos traza y reforestadas con distintas especies leñosas (ver objetivos en **Capítulo 2**) han sido realizados en el Corredor Verde del Guadiamar, un Paisaje Protegido localizado en el sur-oeste de la Península Ibérica, a 25 km de distancia de la ciudad de Sevilla, creado tras el desastre minero de Aznalcóllar. Desde el accidente minero, la dinámica de los elementos traza y las consecuencias medioambientales, tanto de la contaminación como de las medidas de remediación, han sido estudiadas en esta zona obteniéndose numerosos y valiosos resultados.

3.1 Accidente minero de Aznalcóllar

El complejo minero de Aznalcóllar se encuentra situado en el sureste de la faja pirítica ibérica, la cual constituye la mayor y más importante concentración de sulfuros masivos de Europa Occidental. La faja pirítica ibérica está localizada en el suroeste de la Península Ibérica extendiéndose unos 200 km desde el suroeste de Portugal hasta el oeste de Sevilla, en España. Los principales metales obtenidos en esta explotación son Zn, Cu, Pb y Ag.

En este complejo se construyó una balsa de decantación para almacenar los residuos procedentes de la molienda y flotación del mineral. Este depósito con una superficie de unas 200 ha y capacidad final proyectada de 33 hm^3, comenzó a utilizarse en 1979; según aumentaba su llenado, se llevaron a cabo diferentes etapas de recrecimiento del dique hasta 1997 (Gómez de las Heras et al., 2001).

En la madrugada del 25 de abril de 1998 uno de los muros de contención de la balsa de decantación sufrió una rotura de unas dimensiones de 60 m de ancho y 30 m de alto, vertiendo unos 5.5 hm^3 de lodos tóxicos y 1.9 hm^3 de aguas ácidas. Alrededor de 20 hm^3 de materiales tóxicos permanecieron en la balsa (Arenas et al., 2001).

Este vertido alcanzó al río Agrio, ya que apenas le separaban 200 m del punto de salida, el cual desagua en el río Guadiamar. El vertido se extendió 60 km de longitud a lo largo de los cauces y las llanuras aluviales de los ríos Agrio y Guadiamar, con una anchura de hasta 2 km (media de 500 m). Los lodos se fueron depositando a lo largo del río hasta la zona de Entremuros, mientras que las aguas ácidas fueron circulando hasta el río Guadalquivir. Tras el vertido, se aplicaron unas medidas urgentes para contener el avance

de la contaminación, como fueron la paralización de la actividad minera en Aznalcóllar, explotada entonces por la empresa Boliden Apirsa, S.L., y el almacenamiento de las aguas ácidas en Entremuros, para evitar que la contaminación llegara al Parque Nacional de Doñana y al estuario del río Guadalquivir. La actividad agrícola se suprimió y se destruyeron las cosechas afectadas por el vertido (Arenas et al., 2001).

Las aguas ácidas que se vertieron estaban cargadas de elementos traza (de mayor a menor concentración): Zn, Mn, Pb, Ni, Cd, As, Cr, Cu y Hg (Cabrera et al., 1999). Uno de los efectos de este vertido fue un cambio drástico en las aguas del río (pH cercano a 3 y oxígeno disuelto de 0.5-1 g L^{-1}) que acabó con la vida de todos los organismos acuáticos (Grimalt et al., 1999). Los lodos estaban constituidos principalmente por Fe (34-37 %) y S (25-40 %), procedentes de la pirita, y por numerosos elementos traza (de mayor a menor concentración, en porcentaje): Pb, Zn, As, Cu, Sb, Co, Tl, Bi, Cd, Ag, Hg y Se (Grimalt et al., 1999; P. Madejón et al., 2018b).

3.2 Restauración y creación del Corredor Verde del Guadiamar

La primera actuación prioritaria fue la retirada de los lodos, debido al riesgo de salud pública y de contaminación ambiental, así como de la capa superficial del suelo afectado (5-20 cm) (Cabrera et al., 2005). El objetivo fue finalizar la limpieza antes de la llegada del otoño de 1998 y las lluvias para evitar que la previsible crecida del río Guadiamar desplazara la contaminación al Parque Nacional de Doñana. Los espesores del lodo fueron variables (desde varios mm hasta más de 1 m), concentrándose en el sector desde la mina hasta el puente de Las Doblas, con el 62% del volumen total de lodos (1560 m^3 ha^{-1}); mientras que un 38 % (400-500 m^3 ha^{-1}) se extendió por el sector Doblas-Entremuros (ITGE, 1998).

La retirada de lodos comenzó en mayo de 1998 y finalizó en diciembre de 1998, aunque se tuvo que realizar una segunda limpieza, finalizada en febrero de 2000. Se limpiaron más de 4000 ha y se recogieron 7 hm^3 de lodo y suelo superficial contaminado que fueron vertidos en la Corta de Aznalcóllar (Arenas et al., 2001).

El territorio afectado por los lodos estaba principalmente dedicado a la agricultura de cultivos arbóreos y herbáceos, con zonas de dehesas y pastizales (Carrascal et al., 2008). Por lo tanto, se realizó la expropiación y compra de los terrenos afectados por los lodos

para evitar el riesgo de toxicidad por el consumo de alimentos procedentes del uso agrícola y ganadero del suelo contaminado (Serrano, 2000).

Tras la limpieza de lodos y del suelo superficial contaminado, se añadieron diferentes tipos de enmiendas a diferentes concentraciones según las propiedades de cada suelo. El principal objetivo de la adición de enmiendas fue la disminución de la movilidad y biodisponibilidad de los elementos traza del suelo. También con la aplicación de estas enmiendas se conseguía mejorar la fertilidad de los suelos (Montiel-Rozas et al., 2018). Primero se añadió espuma azucarera, una enmienda caliza para la corrección de suelos ácidos, a una concentración de 15-40 Tn ha^{-1}, entre febrero y octubre de 1999. Después se añadió materia orgánica en forma de estiércol y compost a una concentración de 15-20 Tn ha^{-1} entre octubre y diciembre de 1999, con la finalidad de aumentar la fertilidad. Por último, puntualmente se procedió a la aplicación de arcilla entre diciembre de 1999 y junio de 2001 a una concentración muy variable, 320-960 Tn ha^{-1}, para retener los elementos traza por adsorción o en el complejo de cambio de las arcillas (REDIAM, 2002). Tras la aplicación de las enmiendas se homogeneizaron los primeros 25 cm del suelo mediante una labor de grada (Cabrera et al., 2005).

Finalizada la fase de limpieza y aplicación de enmiendas, se comenzó con la restauración de la zona afectada dentro del Proyecto del Corredor Verde del Guadiamar. Con este proyecto se pretendía recuperar la funcionalidad del sistema fluvial y de la cuenca del Guadiamar, a través de la conexión de los sistemas naturales protegidos de Sierra Morena y los Parques Natural y Nacional de Doñana (Secretaría General Técnica, 1999). En los suelos recuperados se llevó a cabo un proyecto de "restauración ecológica", con el objetivo de reconstruir las comunidades vegetales previas a la intervención humana (Serrano, 2000).

La forestación tuvo lugar en los años 1999 y 2000 y la densidad de especies leñosas alcanzó 700-900 plantas ha^{-1} (Domínguez et al., 2010b). Se aplicaron seis modelos básicos de vegetación y tres de transición, en los que se utilizaron unas 40 especies de árboles y matorrales, empleando un total de más de tres millones de plantones (Cabrera et al., 2005; P. Madejón et al., 2018a).

En 2003, se declaró el Paisaje Protegido Corredor Verde del Guadiamar, con una superficie de 2706 ha bajo la titularidad de la Comunidad Autónoma de Andalucía. Con este nuevo espacio protegido se recuperaba una de las funciones de la cuenca del Guadiamar: servir de conexión entre la sierra y los sistemas litorales. Se definieron una

serie de actividades no compatibles con la conservación del espacio, prohibiendo recolectar o capturar especies de fauna y flora silvestre, así como la actividad cinegética y la pesca (Consejería de Medio Ambiente, 2003).

3.3 Evolución de la restauración

Numerosos estudios han sido realizados desde el vertido minero para evaluar la evolución de la restauración de la zona (revisados en P. Madejón et al. (2018b)). Tras la limpieza de lodos, diferentes estudios encontraron valores de elementos traza en los suelos más altos que los valores de fondo, y una gran heterogeneidad en los niveles de contaminación (Cabrera et al., 2008, 2005; Domínguez et al., 2016; Galán et al., 2002; Simón et al., 2008). Estos resultados indicaron que la limpieza de lodos no fue completa y que los restos de lodo se han incorporado al suelo al añadir las enmiendas en determinadas zonas, especialmente en la zona norte del Corredor más afectada por el vertido y con unas propiedades de suelo menos favorables para amortiguar la movilidad de los elementos traza del suelo (Alonso et al., 2001; Cabrera et al., 2005). Los estudios extensivos de seguimiento de la contaminación en el Corredor han sido complementados con estudios de monitorización de parcelas experimentales, en los que se ha analizado de forma más detallada el efecto a largo plazo de diferentes enmiendas en el suelo. Estos estudios muestran una recuperación natural de los suelos a lo largo de los años, potenciada por la adición de enmiendas calizas y orgánicas, que propician un aumento del pH del suelo y del contenido de carbono orgánico, reduciendo la concentración de la fracción biodisponible de los elementos traza (E. Madejón et al., 2009, 2006; P. Madejón et al. 2018a; Xiong et al., 2015)

La forestación con especies de árboles y arbustos no registró la misma supervivencia y crecimiento en todo el Corredor. Carrascal et al. (2008) establecieron tres áreas con diferentes características, en cuanto al desarrollo de la vegetación: el tramo norte, desde la balsa al puente de Las Doblas, el tramo centro hasta el Vado del Quema y el tramo sur, hasta el límite de Doñana. En el tramo norte el desarrollo de la vegetación fue escaso (62% del terreno catalogado como sin/ baja cobertura), debido a su mayor afección por el vertido y por poseer suelos menos desarrollados y con mayores concentraciones de elementos traza. En el tramo centro, ocupado por una llanura aluvial con suelos ricos en

materia orgánica y humedad, la vegetación estuvo más desarrollada; más de 25 ha fueron catalogadas como con cobertura vegetal muy buena, aunque el desarrollo de la vegetación herbácea dificultaba el crecimiento y supervivencia de los plantones forestados. El tramo sur estaba dominado por la antesala de la marisma, presentaba un 90% de cobertura media o buena y con bosques de ribera en estado óptimo de desarrollo. Durante los primeros años, se produjo una alta mortalidad de los plantones debido a numerosos factores como la contaminación, la sequía, la alteración de la estructura edáfica y la competición con herbáceas (Domínguez et al., 2010a).

3.4 Características del suelo y climatología

El Corredor Verde del Guadiamar comienza al norte del complejo minero de Aznalcóllar, localizado en la faja pirítica, y discurre por los cauces y las llanuras aluviales de los ríos Agrio y Guadiamar hasta Entremuros, en las marismas del Guadalquivir. El río Agrio es el principal afluente del Guadiamar, a su vez el Guadiamar es el último gran afluente de la margen derecha del río Guadalquivir, antes de su desembocadura en el Océano Atlántico. La cuenca del Guadalquivir se formó durante el Neógeno y el relleno sedimentario se realizó cuando se estructuraba la Cordillera Bética, entre el Mioceno inferior y el Plioceno (Villalobos Megía y Pérez Muñoz, 2006). Los depósitos aluviales del río Guadiamar están compuestos por limos, arenas y gravas (López-Pamo et al., 1999). Los suelos son muy heterogéneos debido a una litología y geomorfología muy variada.

En el tramo norte, desde la mina hasta el puente de Las Doblas (los primeros 15 km), el lecho del río está compuesto de arenas y gravas, la ribera con depósitos de arenas medias-finas y la llanura aluvial (350- 1000 m de ancho) está formada por grava y arenas medias-gruesas. El tramo central discurre desde el km 15 al 30 (desde la mina); el lecho del río está formado por guijarros y arena. En este tramo, en la llanura aluvial (300-700 m de ancho) se forman pequeños diques naturales debido a los sedimentos que deja el río con textura de arena y limo. En el tramo sur, desde el Vado del Quema hasta Entremuros, el lecho del río está dominado por los sedimentos de la marisma con textura de arena fina y limo. Este tramo se abre a un canal con un dique artificial que previene las inundaciones (Gallart et al., 1999).

De acuerdo con la taxonomía de suelos de la USDA (United States Department of Agriculture) los suelos de la llanura aluvial del río Guadiamar podrían clasificarse como Typic y Aquic Xerofluvents calcáreos y no-calcáreos con texturas arenosa y arenosa-franca. Los suelos de las terrazas bajas son Typic y Aquic Haploxeralfs y Aquic Xerofluvents, mientras que en las terrazas altas son Typic Rhodoxeralf y Pseudogley. Los suelos entre las terrazas y la llanura aluvial son Calcixerollic Xerochrepts y están erosionados (Cabrera et al., 1999; P. Madejón et al., 2018b).

El clima es Mediterráneo, con inviernos lluviosos y moderadamente fríos, y con veranos secos y calurosos. La temperatura media anual es de 17 ºC (mínima de 5.2 ºC en enero y máxima de 33.5 ºC en julio) y la precipitación media anual es de 500 mm (P. Madejón et al., 2018a).

3.5 Zonas de muestreo y especies arbóreas seleccionadas

3.5.1. Zonas y parcelas de muestreo

El Corredor Verde del Guadiamar ocupa una superficie de 2706 ha y más de 60 km de longitud, a lo largo de los márgenes de los ríos Agrio y Guadiamar. El vertido afectó a zonas con diferentes características geológicas y edáficas que fueron remediadas con los mismos criterios y representan una oportunidad como laboratorio natural donde evaluar una serie de preguntas relativas a la evolución de los ecosistemas emergentes en función de las características edáficas de partida. Debido a su gran heterogeneidad geomorfológica e hidrológica (Gallart et al., 1999), se distinguieron tres tramos – norte, centro y sur – en la planificación de la restauración (Carrascal et al., 2008). El tramo sur corresponde a la canalización del río (Entremuros) y está afectado por las marismas salobres, por tanto no ha sido incluido en este estudio de suelo-árbol-microorganismos. En esta tesis doctoral se ha estratificado el muestreo en dos zonas contrastadas: la “zona norte” (desde la mina de Aznalcóllar hasta la altura con Sanlúcar la Mayor, que coincide con el tramo norte) y la “zona sur” (desde el paso del río Guadiamar por Aznalcázar hasta el término municipal de Villamanrique de la Condesa, que coincide con el tramo central), con el fin de comparar las interacciones suelo-planta-microorganismo en dos zonas con características edáficas tan diversas (Fig. 3.1). La “zona norte” se caracteriza por tener suelos naturalmente ácidos dominados por pizarras y esquistos con textura franco-

arenosa, mientras en la "zona sur" los suelos son neutros-básicos con textura franca, dominados por calizas y calcarenitas (Domínguez et al., 2008).

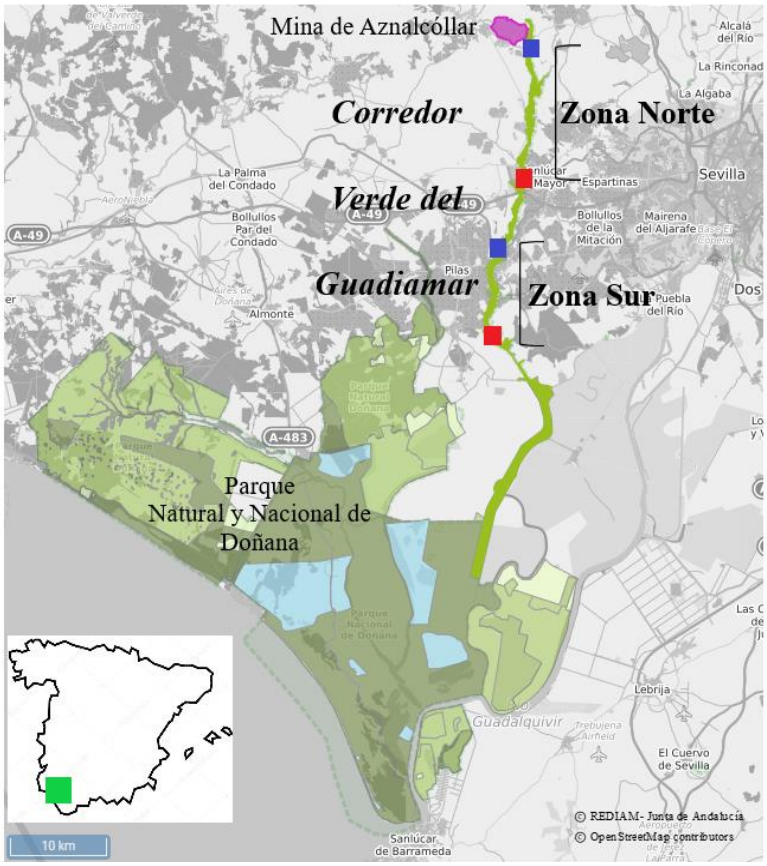


Figura 3.1 Mapa de localización del Corredor Verde del Guadiamar, con las zonas y parcelas de muestreo. En azul las parcelas no afectadas y en rojo las parcelas afectadas por el vertido.

Aparte de las diferencias geológicas, los niveles de contaminación a lo largo del área de estudio son desiguales. La "zona norte" registra unos niveles de contaminación mayores que la "zona sur" debido, entre otras causas, a su mayor proximidad a la mina. Las labores de limpieza fueron más agresivas en la "zona norte" que en la "zona sur", debido a la mayor acumulación de lodos en el norte (Domínguez et al., 2016). Sin embargo, la limpieza de los lodos no fue homogénea y parte de los suelos siguen estando altamente contaminados y degradados, con el problema adicional de que los niveles de contaminación son muy variables en distancias cortas debido a la deposición irregular de los lodos (García-Carmona et al., 2019a). Estos restos de lodos, ricos en pirita, promueven la acidificación del suelo lo cual conlleva una mayor solubilidad de elementos traza, por lo tanto, una mayor movilidad en el ecosistema (Domínguez et al., 2016). Esta

acidificación se produce especialmente en la "zona norte", donde los suelos son más arenosos y apenas contienen carbonatos, por lo que la capacidad tamponadora de estos suelos es reducida, en comparación con los suelos de la "zona sur" donde la textura es franca y hay un alto contenido en carbonatos, favoreciendo la inmovilización de los elementos traza en la matriz del suelo.

Se han seleccionado dos parcelas de muestreo, una en cada zona mencionada. La parcela norte ocupa 21.2 ha y la parcela sur 5.7 ha y, en general, estas parcelas han evolucionado favorablemente a las medidas de remediación aplicadas (Fig. 3.2).

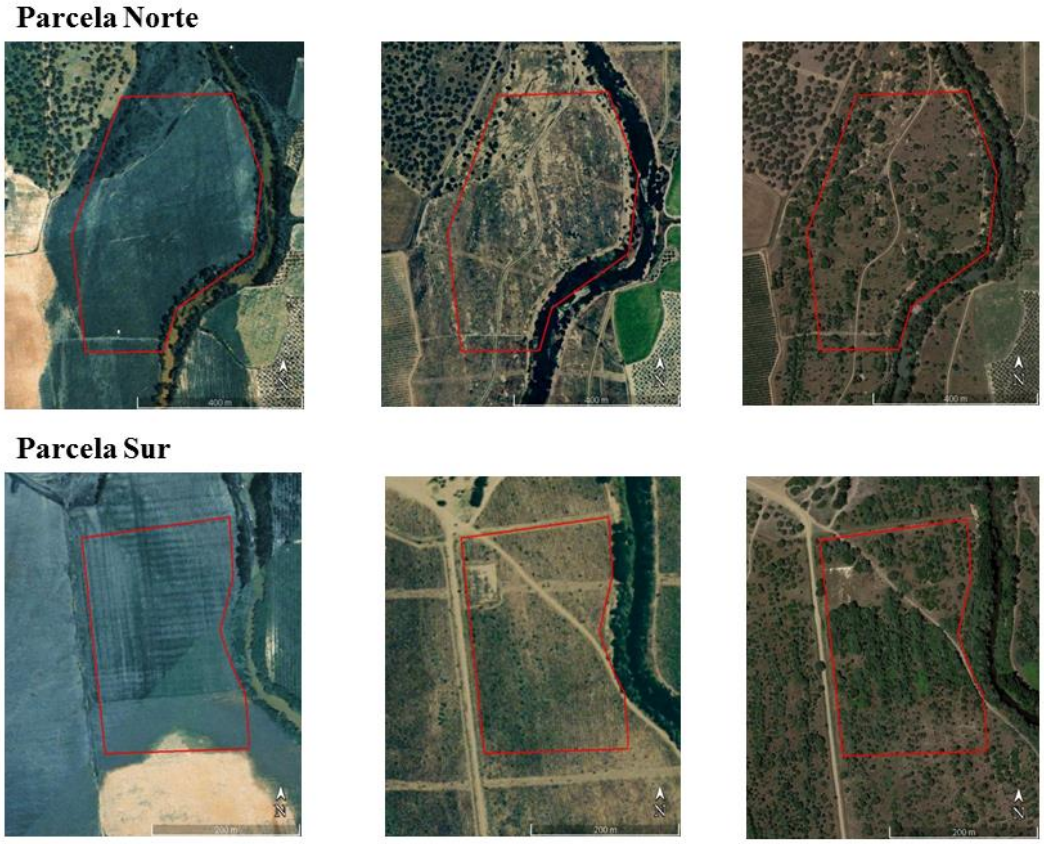


Figura 3.2 Ortofotos de la evolución de la restauración de las dos parcelas de muestreo en los años 1998, 2004 y 2018 (de izquierda a derecha). Las parcelas de muestreo están demarcadas en rojo. Fuente: GoogleEarth 2019 y REDIAM 2020.

Estas parcelas presentan características edáficas y niveles de contaminación residual contrastados, y a la vez presentan especies leñosas comunes (introducidas en los mismos marcos de plantación durante la restauración de la zona) con un grado de desarrollo similar, lo cual permitirá analizar el efecto de la identidad de estas especies leñosas en procesos físico-químicos y biológicos del suelo bajo diferentes contextos edáficos.

La aplicación de enmiendas durante las labores de restauración no fue homogénea y varió según la contaminación de los suelos. En la "zona norte", se añadieron enmiendas de materia orgánica (20 Tn ha^{-1}), calizas (20 Tn ha^{-1}), y arcillas (720 Tn ha^{-1}). Mientras que en la "zona sur", se añadieron enmiendas de materia orgánica (15 Tn ha^{-1}) y calizas (20 Tn ha^{-1}), pero no se añadieron arcillas debido a la textura franca de estos suelos (Fig. 3.3).

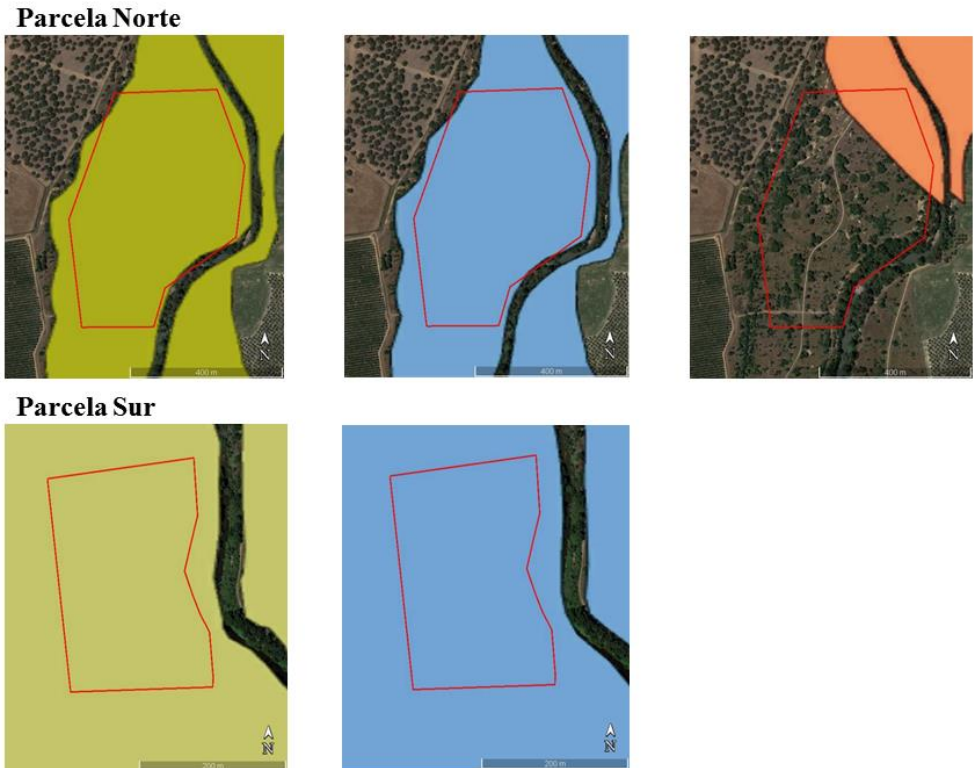


Figura 3.3 Enmiendas aplicadas en las áreas de estudio de las zonas norte y sur. Enmiendas de materia orgánica (verde), calcáreas (azul) y arcilla (naranja). Fuente: GoogleEarth 2019 y REDIAM 2020.

Para el estudio de las comunidades de hongos ectomicorrícicos asociados a la encina (**Capítulos 6 y 7**), se seleccionaron además dos parcelas (una en cada zona) que no habían sido afectadas directamente por el vertido (Fig. 3.1). En estas parcelas, incluidas en el Corredor Verde, se aplicaron las mismas técnicas de remediación y en este caso, se plantaron también encinas (*Quercus ilex*) facilitando así el estudio comparativo.

Como se ha mencionado, las parcelas de muestreo fueron seleccionadas según su geología y nivel de contaminación del suelo, y también fue importante elegir aquellas áreas donde las especies forestales de estudio se habían establecido y crecido con éxito. En los

respectivos capítulos se presentarán más detalles de las características físico-químicas y biológicas del suelo en las parcelas de muestreo.

3.5.2. Especies arbóreas de estudio y tipos de micorrizas

Las cuatro especies arbóreas seleccionadas para el estudio han sido ampliamente usadas durante el programa de restauración del Corredor Verde del Guadiamar y tienen atributos funcionales y ecológicos contrastados.

- El acebuche (*Olea europaea* subsp. *europaea* var. *sylvestris* (Mill.) Lehr) es una especie perennifolia y longeva, que ha sido cultivada de manera extensiva en la cuenca del mar Mediterráneo (Lumaret et al., 2004). El acebuche puede adaptarse a condiciones ambientales adversas debido a su tolerancia a la sequía, a la salinidad y a las bajas temperaturas. Esta buena adaptabilidad a diferentes condiciones ambientales hace de esta especie una buena candidata para colonizar suelos de zonas degradadas, deforestadas o suelos marginales (Díaz-Rueda et al., 2020).

- El álamo blanco (*Populus alba* L.) es una especie caducifolia de crecimiento rápido muy abundante en los bosques de ribera de la cuenca del mar Mediterráneo, centro de Europa y oeste de Asia (Palancean et al., 2018). Pertenece a la familia Salicaceae y como la mayoría de especies de esta familia, el álamo blanco acumula elementos traza en sus hojas. En el Corredor esta especie ha sido utilizada como bioindicadora de la disponibilidad de Cd y Zn en el suelo (P. Madejón et al., 2004).

- El pino piñonero (*Pinus pinea* L.) es una conífera ampliamente distribuida en la cuenca del mar Mediterráneo. Es de gran importancia económica como recurso maderero y alimentario, por lo que se ha plantado extensivamente en los últimos siglos (Fady et al., 2008).

- La encina (*Quercus ilex* subsp. *ballota*) es un árbol o arbusto perennifolio de hojas anchas que se distribuye principalmente en el centro-oeste de la cuenca del mar Mediterráneo. Es una especie que se desarrolla en una gran variedad de suelos y en un gradiente amplio de climas típicamente Mediterráneos (de Rigo y Caudullo, 2016). Estudios previos de esta especie en el Corredor han mostrado que tienen una alta capacidad para retener Cd en sus raíces, facilitando la fitoestabilización de este elemento traza (Domínguez et al., 2011, 2009).

Las especies arbóreas estudiadas se caracterizan por formar asociaciones simbióticas con los hongos, en forma de micorrizas. Existen dos tipos principales de micorrizas que pueden establecerse con especies arbóreas; las micorrizas arbusculares y las ectomicorrizas. El acebuche se asocia con los hongos micorrícicos arbusculares, el álamo blanco y la encina se asocian con ambos tipos micorrizas, mientras que el pino piñonero se asocia con ambos pero principalmente con los hongos ectomicorrícicos (Maremmani et al., 2003; Navarro-Fernández et al., 2016).

3.6 Bibliografía

Alonso, C., Antón Pacheco, C., Barettino Fraile, D., Cabrera, F., Fernández, A.M., Fernández, J.E., García Gutiérrez, M., Giráldez Cervera, J. V, Girón Moreno, I.F., López Pamo, E., Moreno, F., Ordóñez, R., Pelayo, M., Rivas, P., Vanderlinden, K., Villar, M. V, 2001. Los suelos del Guadiamar: estudios de caracterización y de la evolución de los suelos contaminados por el lodo. Boletín geológico y minero Especial, 163-198.

Arenas, J.M., Carrero, G., Galache, J., Mediavilla, C., Silgado, A., Vázquez, E.M., 2001. Actuaciones realizadas tras el accidente de Aznalcóllar. Boletín geológico y minero Especial, 35-56.

Cabrera, F., Ariza, J., Madejón, P., Madejón, E., Murillo, J.M., 2008. Mercury and other trace elements in soils affected by the mine tailing spill in Aznalcóllar (SW Spain). Science of the Total Environment 390, 311-322.

Cabrera, F., Clemente, L., Díaz Barrientos, E., López, R., Murillo, J.M., 1999. Heavy metal pollution of soils affected by the Guadiamar toxic flood. Science of the Total Environment 242, 117-129.

Cabrera, F., Murillo, J.M., Madejón, P., Domínguez, M.T., Burgos, P., Madejón, E., Marañón, T., Pérez de Mora, A., Cordón, R., Moreno, F., Girón, I., 2005. Los suelos del Guadiamar: contaminación por elementos traza tras el accidente minero de Aznalcóllar, en: Consejería de Medio Ambiente (Ed.), La Restauración Ecológica del Río Guadiamar y el Proyecto del Corredor Verde. Junta de Andalucía, pp. 145-164.

Carrascal, F., Gil, A., Montes, C., Pérez de Guzmán, R., 2008. La vegetación en la cuenca del Guadimar y en el Corredor Verde, en: Consejería de Medio Ambiente (Ed.), La Restauración Ecológica del Río Guadiamar y el Proyecto del Corredor Verde. Junta de Andalucía, pp. 283-306.

Consejería de Medio Ambiente, 2003. Decreto 112/2003, de 22 de abril, por el que se declara Paisaje Protegido el Corredor Verde del Guadiamar. España.

de Rigo, D., Caudullo, G., 2016. Quercus ilex in Europe: distribution, habitat, usage and threats, en: San-Miguel-Ayanz, J., de Rigo, D., Caudullo, G., Houston Durrant, T., Mauri, A. (Eds.), European Atlas of Forest Tree Species. Publ. Off. EU, Luxembourg, pp. 152-153.

Díaz-Rueda, P., Franco-Navarro, J.D., Messora, R., Espartero, J., Rivero-Núñez, C.M., Aleza, P., Capote, N., Cantos, M., García-Fernández, J.L., de cires, A., Belaj, A., León, L., Besnard, G., Colmenero-Flores, J.M., 2020. SILVOLIVE, a germplasm collection of wild subspecies with high genetic variability as a source of rootstocks and resistance genes for olive breeding. Frontiers in Plant Science 11, 1-15.

Domínguez, M.T., Alegre, J.M., Madejón, P., Madejón, E., Burgos, P., Cabrera, F., Marañón, T., Murillo, J.M., 2016. River banks and channels as hotspots of soil pollution after large-scale remediation of a river basin. Geoderma 261, 133-140.

Domínguez, M.T., Madejón, P., Marañón, T., Murillo, J.M., 2010a. Afforestation of a trace-element polluted area in SW Spain: woody plant performance and trace element accumulation. European Journal of Forest Research 129, 47-59.

Domínguez, M.T., Madrid, F., Marañón, T., Murillo, J.M., 2009. Cadmium availability in soil and retention in oak roots: potential for phytostabilization. Chemosphere 76, 480-486.

Domínguez, M.T., Marañón, T., Murillo, J.M., Redondo-Gómez, S., 2011. Response of Holm oak (Quercus ilex subsp. ballota) and mastic shrub (Pistacia lentiscus L.) seedlings to high concentrations of Cd and Tl in the rhizosphere. Chemosphere 83, 1166-1174.

Domínguez, M.T., Marañón, T., Murillo, J.M., Schulin, R., Robinson, B.H., 2010b. Nutritional status of mediterranean trees growing in a contaminated and remediated area. Water, Air, and Soil Pollution 205, 305-321.

Domínguez, M.T., Marañón, T., Murillo, J.M., Schulin, R., Robinson, B.H., 2008. Trace element accumulation in woody plants of the Guadiamar Valley, SW Spain: a large-scale phytomanagement case study. Environmental Pollution 152, 50-59.

Fady, B., Fineschi, S., Vendramin, G., 2008. EUFORGEN Technical Guidelines for genetic conservation and use for Italian stone pine (Pinus pinea). Rome, Italy.

Galán, E., González, I., Fernández-Caliani, J.C., 2002. Residual pollution load of soils impacted by the Aznalcóllar (Spain) mining spill after clean-up operations. Science of the Total Environment 286, 167-179.

Gallart, F., Benito, G., Martín-Vide, J.P., Benito, A., Prió, J.M., Regüés, D., 1999. Fluvial geomorphology and hydrology in the dispersal and fate of pyrite mud particles released by the Aznalcóllar mine tailings spill. Science of the Total Environment 242, 13-26.

García-Carmona, M., García-Robles, H., Turpín Torrano, C., Fernández Ondoño, E., Lorite Moreno, J., Sierra Aragón, M., Martín Peinado, F.J., 2019. Residual pollution and vegetation distribution in amended soils 20 years after a pyrite mine tailings spill (Aznalcóllar, Spain). Science of the Total Environment 650, 933-940.

Gómez de las Heras, J., Gumiel, P., Grima, J., 2001. Contribuciones al estudio de la rotura de la balsa de lodos de Aznalcóllar. Boletín Geológico y Minero Especial, 283-294.

Grimalt, J.O., Ferrer, M., MacPherson, E., 1999. The mine tailing accident in Aznalcollar. Science of the Total Environment 242, 3-11.

ITGE, 1998. Cartografía y ubicación de los lodos mineros en la cuenca del río Guadiamar. Aznalcóllar-Entremuros. Madrid.

López-Pamo, E., Barettino, D., Antón-Pacheco, C., Ortiz, G., Arránz, J.C., Gumiel, J.C., Martínez-Pledel, B., Aparicio, M., Montouto, O., 1999. The extent of the Aznalcóllar pyritic sludge spill and its effects on soils. Science of the Total Environment 242, 57-88.

Lumaret, R., Ouazzani, N., Michaud, H., Vivier, G., Deguilloux, M.F., Di Giusto, F., 2004. Allozyme variation of oleaster populations (wild olive tree) (Olea europaea L.) in the Mediterranean Basin. Heredity 92, 343-351.

Madejón, E., De Mora, A.P., Felipe, E., Burgos, P., Cabrera, F., 2006. Soil amendments reduce trace element solubility in a contaminated soil and allow regrowth of natural vegetation. Environmental Pollution 139, 40-52.

Madejón, E., Madejón, P., Burgos, P., Pérez de Mora, A., Cabrera, F., 2009. Trace elements, pH and organic matter evolution in contaminated soils under assisted natural remediation: a 4-year field study. Journal of Hazardous Materials 162, 931-938.

Madejón, P., Domínguez, M.T., Gil-Martínez, M., Navarro-Fernández, C.M., Montiel-Rozas, M.M., Madejón, E., Murillo, J.M., Cabrera, F., Marañón, T., 2018a. Evaluation of amendment addition and tree planting as measures to remediate contaminated soils: the Guadiamar case study (SW Spain). Catena 166, 34-43.

Madejón, P., Domínguez, M.T., Madejón, E., Cabrera, F., Marañón, T., Murillo, J.M., 2018b. Soil-plant relationships and contamination by trace elements: a review of twenty years of experimentation and monitoring after the Aznalcóllar (SW Spain) mine accident. Science of the Total Environment 625, 50-63.

Madejón, P., Marañón, T., Murillo, J.M., Robinson, B., 2004. White poplar (*Populus alba*) as a biomonitor of trace elements in contaminated riparian forests. Environmental Pollution 132, 145-155.

Maremmani, A., Bedini, S., Matoševic, I., Tomei, P.E., Giovannetti, M., 2003. Type of mycorrhizal associations in two coastal nature reserves of the Mediterranean basin. Mycorrhiza 13, 33-40.

Montiel-Rozas, M.M., Domínguez, M.T., Madejón, E., Madejón, P., Pastorelli, R., Renella, G., 2018. Long-term effects of organic amendments on bacterial and fungal communities in a degraded Mediterranean soil. Geoderma 332, 20-28.

Navarro-Fernández, C.M., Pérez-Ramos, I.M., de la Riva, E.G., Vera, J.R., Roumet, C., Villar, R., Marañón, T., 2016. Functional responses of Mediterranean plant communities to soil resource heterogeneity: a mycorrhizal trait-based approach. Journal of Vegetation Science 27, 1243-1253.

Palancean, I., Alba, N., Sabatti, M., Vries, S.M.G. De, 2018. EUFORGEN Technical guidelines for genetic conservation and use for white poplar (*Populus alba*).

REDIAM, 2002. WMS Enmiendas realizadas en los suelos contaminados del río Guadiamar a escala 1:10.000.

Secretaría General Técnica, 1999. La estrategia del corredor verde del Guadiamar: fundamentos de la estrategia, en: Consejería de Medio Ambiente (Ed.), Seminario Internacional sobre Corredores Ecológicos. Junta de Andalucía, Sevilla.

Serrano, J., 2000. El Corredor Verde del Guadiamar, dos años después del vertido de las minas de Aznalcóllar. Medio Ambiente 34.

Simón, M., García, I., Martín, F., Díez, M., del Moral, F., Sánchez, J.A., 2008. Remediation measures and displacement of pollutants in soils affected by the spill of a pyrite mine. Science of the Total Environment 407, 23-39.

Villalobos Megía, M., Pérez Muñoz, A.B., 2006. Cuenca del Guadalquivir, en: Consejería de Medio Ambiente. Junta de Andalucía (Ed.), Geodiversidad y patrimonio geológico de Andalucía. Itinerario Geológico por Andalucía. Guía didáctica de campo. pp. 275-296.

Xiong, J., Madejon, P., Madejon, E., Cabrera, F., 2015. Assisted natural remediation of a trace element-contaminated acid soil: an eight-year field study. Pedosphere 25, 250-262.

4. COMUNIDADES MICROBIANAS DEL SUELO: BIOMASA, ACTIVIDADES ENZIMÁTICAS Y RESPIRACIÓN

Resumen

Las especies de árboles utilizadas en fitorremediación afectan a las diferentes propiedades del suelo como son el pH, el contenido en materia orgánica y la estructura del suelo, produciendo cambios indirectos en las comunidades microbianas. Estas comunidades microbianas que se establecen en los suelos tienen un papel esencial en el ciclado de nutrientes y en la descomposición de compuestos orgánicos complejos, los cuales son capaces de mineralizar a través de sus actividades enzimáticas. Las condiciones adversas como la contaminación por elementos traza causan una reducción de la biomasa microbiana y de la eficiencia en la utilización de los sustratos. Potencialmente podrían conllevar un descenso de la actividad enzimática y cambios en la estructura de la comunidad microbiana, ya que la microbiota es indicador sensible del estrés y las perturbaciones en el ecosistema. La contaminación del suelo podría suponer una reducción en el ciclado de nutrientes y en el secuestro de carbono.

En este estudio pretendemos evaluar la influencia de la identidad de la especie arbórea en algunos índices que miden la actividad microbiana en un suelo contaminado por elementos traza, que fue forestado como parte de las medidas de fitorremediación. Recogimos muestras de suelo bajo tres especies leñosas (acebuche, álamo blanco y pino piñonero) y en suelos de pradera, en dos sitios con diferentes propiedades edáficas. El muestreo se realizó en primavera y otoño de 2017. En estos suelos se analizaron el carbono y nitrógeno microbianos, la actividad microbiana total, las actividades enzimáticas relacionadas con los ciclos de carbono, nitrógeno y fósforo, y la diversidad catabólica microbiana.

La forestación produjo un aumento en la mayoría de los indicadores microbianos. También se descubrieron efectos específicos de las especies estudiadas en la materia orgánica del suelo con consecuencias en la biomasa y la actividad total microbianas. El álamo blanco fue la especie que promovió una mayor diversidad catabólica lo que podría suponer una mayor mineralización de sustratos simples y complejos de carbono en estos suelos. Las actividades enzimáticas fueron buenas indicadoras del estrés producido por la baja humedad del otoño. Parece que la sequía del otoño tuvo un importante efecto en las comunidades microbianas, ya que se produjo un cambio en los procesos microbianos; las comunidades en primavera estaban altamente determinadas por las propiedades del suelo, mientras en otoño estas comunidades eran más estocásticas. La actividad microbiana total fuer dependiente del sitio, el cual estaba principalmente caracterizado por diferentes niveles de pH y concentraciones de elementos traza.

Abstract

Tree species in phytoremediated soils influence properties such as pH, soil organic matter (SOM) and soil structure, driving indirect changes on soil microbial communities and functionality. **S**oil microbial communities have an essential role in nutrient cycling and in the decomposition of complex organic compounds which are mineralized through their enzyme activities. Adverse conditions such as trace element contamination cause a reduction of soil microbial biomass and efficiency in substrate utilisation, potentially decreasing the production of enzymes and provoking changes in community structure, as microbes are sensitive indicators of stress and disturbances in ecosystem functioning. These effects may impair nutrient cycling and C sequestration in contaminated soils.

In this work, we aimed to evaluate the influence of tree identity on some key indices of microbial activity in soils contaminated by trace elements, which were afforested as part of a large phytoremediation programme. We sampled soils under three afforested tree species (wild olive, white poplar and stone pine) and under grasslands at two sites with contrasting soil conditions. We repeated the sampling in two seasons, spring and autumn 2017. We determined soil microbial biomass, total microbial activity, enzyme activities (involved in C, N and P cycles) and microbial catabolic diversity.

Tree afforestation increased most of the measured microbial indicators. This study revealed a tree species-specific effect on soil organic matter with consequences for soil microbial biomass and total microbial activity. Among tree species, white poplar was promoting the highest catabolic diversity in the soil microbial community, enhancing the mineralization of both simple and complex C compounds. Microbial enzyme activities were good indicators of a seasonal drought stress. The unusual drought in autumn had important effects on microbial communities, shifting from deterministic to stochastic microbial processes. Soil pH and trace element concentrations were key variables influencing microbial activity, which tend to exhibit site-dependent patterns; therefore, comparison of soil microbial activities between sites with different pH must be carefully interpreted.

4.1 Introduction

Microbial communities play a key role in biogeochemical cycling and energy flows on terrestrial ecosystems. The composition of soil microbial communities is strongly determined by plant communities, mainly through the effects of rhizodeposition and the decay of leaf litter and roots (Nannipieri et al., 2017). Dominant tree species can influence different soil properties such as pH, soil organic matter (SOM) and soil structure (Ayres et al., 2009) and these changes indirectly affect soil microbial communities (Prescott and Grayston, 2013). Therefore, the links between microbial and plant communities can determine key ecosystem processes which are essential for ecosystem multifunctionality (Grosso et al., 2018).

Although soil quality depends on complex properties, microbial and biochemical characteristics are used as indicators of soil functioning due to their role in nutrient cycling and their susceptibility to changes (Nannipieri et al., 2017). Soil quality is mainly determined by the composition of the SOM, often measured by the abundance of organic substances with different turn-over rates and the soil C:N ratio, which may determine total microbial biomass as well as microbial enzyme activities and catabolic diversity. These microbial processes will affect the cycling of nutrients and the rates of SOM decomposition, eventually determining the rates of C sequestration in the soil (Pérez-Izquierdo et al., 2018).

Among other functions, soil microbial communities have an essential role in nutrient cycling and in the decomposition of complex organic compounds mediated by microbial enzyme activities (Baldrian, 2014). These enzyme activities are very sensitive to stress and disturbances and are considered effective indicators of soil functional changes (Quilchano and Marañón, 2002). Enzyme activities are influenced by both soil abiotic (pH, C, N, P, temperature and moisture) and biotic (microbial biomass and other enzymes) variables (Sinsabaugh et al., 2008). After enzymatic degradation, these products are used by microbes for metabolism and growth (German et al., 2011).

Trace elements are known to be toxic to most organisms when present in excessive concentrations. In contaminated soils, microorganisms and their processes may be disturbed by high concentrations of trace elements producing severe ecosystem perturbations (Giller et al., 1998). Previous studies have shown that trace element toxicity

produces a strong decrease in soil microbial biomass (Barajas-Aceves, 2005; Kandeler et al., 2000), which might result in a reduction in the efficiency of substrate utilisation (Chander and Joergensen, 2001), a reduction in the production of enzymes (Kandeler et al., 2000) and changes in community structure (Abaye et al., 2005). In addition, microbial communities' shifts could result in altered soil metabolism with consequences in ecosystem processes (Krumins et al., 2015).

Recovery of ecosystems after a contamination event requires the restoration of the positive interactions between plant and soil communities (Krumins et al., 2015). In trace element contaminated soils, microbial communities and aboveground plants respond together to contamination by modifying the soil environment (e.g. by translocation, absorption or sequestration of elements) (Clarholm and Skyllberg, 2013b). If microbial communities fail to adapt or to develop resistance to trace elements, microbial biomass may be reduced, and plant growth could be compromised (Krumins et al., 2015). Phytoremediation aims to reduce contaminants by the use of plants and their associated microorganisms and is considered the most feasible technology for the recovery of large areas contaminated by trace elements (Ali et al., 2017; Mendez and Maier, 2008). The advantages of phytoremediation are multiple; it is a cost-efficient, *in situ* technique, which enhances the aesthetic value of the site, with a potential for restoring overall ecosystem function (Garbisu et al., 2002). The use of trees may be a feasible reclamation solution to avoid the transfer of trace elements to adjacent systems and it could also provide long-term benefits, increasing the functionality of the reclaimed ecosystem (Madejón et al., 2018a). In order to develop maximum functionality within the reclaimed ecosystem, the establishment and survival of planted trees is essential, as well as reaching optimal soil functionality to create a long-term self-sustaining ecosystem.

Physical, chemical and biological changes that take place in the soil after tree afforestation have a major effect on ecosystem functionality (Aponte et al., 2013; Mitchell et al., 2010b). However, not all tree species produce the same effects on soil microbial communities. Plant tissue chemistry determines microbial decomposition rates through its nutrient content and turnover rates, as well as mediates biogeochemical processes such as acidification and nitrification with further impacts on soil pH and soil organic matter stabilisation (Angst et al., 2018; Berg and McClaugherty, 2014; Madejón et al., 2018b, 2018a; Marañón et al., 2015; Orozco-Aceves et al., 2015)

In a contaminated and phytoremediated area in SW Spain, we determined the effects of three different tree species on soil functionality and compared with adjacent treeless grasslands. We determined the rates of some microbial processes to measure soil functions. Soil microbial biomass and enzyme activities, as well as total microbial activity and Community Level Physiological Profiles (CLPPs) were measured 19 years after the implementation of the restoration measures. We conducted the study at two sites, at different distances downstream from the contamination source (a mining tailing dam), and for two different seasons (spring and autumn).

We hypothesized that i) trace element contamination reduces soil microbial biomass and activity; ii) afforestation with different tree species induces variations on soil microbial communities´ activity; iii) the interactions of trees and soil microbes are influenced by site conditions, in particular by soil pH and trace element concentration, and iv) seasonal changes in temperature and soil moisture are partly responsible for variations in soil microbial activity.

We expect that this study would help to understand how specific tree species, used in phytoremediation, could affect microbial processes. This knowledge is useful to help to improve the management of contaminated areas taking into account the most adequate selection of tree species for afforestation.

4.2 Materials and methods

4.2.1 Sample design, collection and processing

We selected two sites of contrasting lithology and geomorphology in the Guadiamar Green Corridor. The North site (37°23'08.85" N, 6°13'41.81" W) and the South site (37°14'35.90" N, 6°15'49.74" W), located at 15 km and 30 km away from the mine tailings spill point, respectively. See the general description of the study site in chapter 3.

Among the shrub and tree species afforested during the reclamation program, we selected three characteristic tree species in the study area, which were well established at both sites and had contrasted life-history strategies: wild olive (*Olea europaea*), white poplar (*Populus alba*) and stone pine (*Pinus pinea*).

At both sites, North and South, soil samples were taken with an auger at 0-5 cm depth in four habitat types, i.e. plant covers. Three habitats were under the canopy of the selected tree species and the fourth habitat was the adjacent treeless areas where natural grassland was established. In each site seven replicates per plant cover were sampled, making a total of 56 sampled soils. Replicated trees were separated by at least 30 m. Soil samples were taken to the laboratory and kept stored overnight at 4 ºC until next day when soil was processed.

At each site we sampled soils at two different seasons, spring (March 2017) and autumn (October 2017), for studying differences in soil communities due to seasonal variations. For each season, mean temperatures were similar between sampling sites with mean values of 13.5 ºC in March and of 21.8 ºC in October (Fig. 4.1). Annual rainfall was low in 2017, with values of accumulated rainfall of 381 and 352 mm in the North and South sites, respectively. October was drier than March at both sites, and there was rainfall scarcity over the summer months. In March, the North site registered a higher rainfall (83 mm) than the South site (57 mm). In October, the tendency changed with 23 mm rainfall at the South site compared to the 15 mm rainfall in the North site (Fig. 4.1).

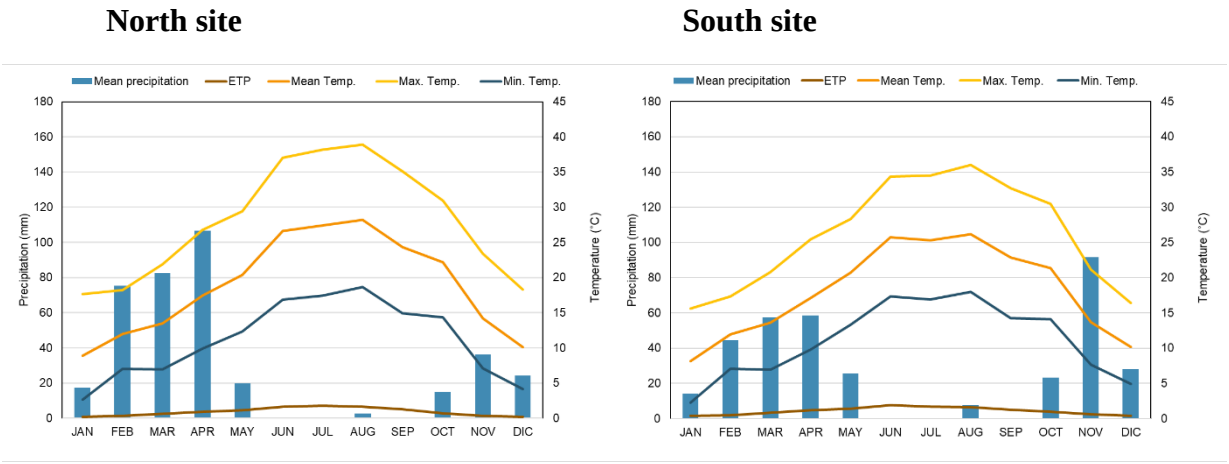


Figure 4.1 Climatic data of year 2017 at the North site (Sanlúcar la Mayor station) and South site (Aznalcázar station).

4.2.2 Soil chemical analyses

A fraction of the soil samples was air-dried and sieved to < 2 mm for physico-chemical analyses. Soil moisture was calculated in both seasons by weight difference between fresh and oven-dried soil after 48 h at 105 ºC. In spring, we measured soil pH, total C and N, carbonate content and P. Soil pH was measured in a 1:2.5 soil- KCl 1 M suspension by using a pH meter (CRISON micropH 2002). Total C and N content was determined using a Flash HT Plus elemental analyser. Carbonate content was measured by the manometric method. The difference between total C and carbonates provided organic C content. Available P was measured by Olsen method (Olsen et al., 1954). In autumn, we measured total soil elements (As, Cr, Cu, Fe, Mn, Pb, S and Zn), using X-Ray fluorescence (XRF) spectroscopy (XRF Niton™).

4.2.3. Soil microbial analyses

A fraction of fresh soil samples was kept at 4 ºC for one week and soil was sieved to < 2 mm for microbial analyses. In both seasons, we determined microbial biomass, enzyme activities and total microbial activity. Microbial biomass was determined by the fumigation-extraction method modified by Gregorich et al. (1990). Microbial C and N were extracted in fumigated and non-fumigated samples with 0.5 M potassium sulphate solution (Vance et al., 1987). Different hydrolytic enzyme assays were carried out to determine the maximum potential enzyme activity in the soil (Table 4.1). Soil enzyme activities, including β-glucosidase (BGL), acid phosphatase, N-acetyl-glucosaminidase and leucyl-aminopeptidase (LAP), were measured using a microplate fluorimetric assay (Marx et al., 2001). The substrates for BGL, ACP and NAG enzymes were conjugates of the fluorescent compound 4-methylumbelliferyl (MUB), and 50 mM sodium acetate buffer was used to maintain an optimal pH of 5.5 during assay (Tabatabai and Bremner, 1969; Tabatabai, 1982). The substrate for LAP activity was L-Leucine-7-amido-4-methylcoumarin (AMC) and 50 mM TRIS buffer was used to maintain an optimal pH of 7.8 for this enzyme. Dehydrogenase (DH) activity was determined in a 1 M TRIS–HCl buffer to maintain an optimal pH of 7.5, after adding 2-p-iodofenil-3p-nitrofenil-5-feniltetrazolio (INT) as an electron acceptor (Trevors, 1984a). The iodonitrotetrazolium

formazan (INTF) produced was measured spectrophotometrically at 490 nm. Fluorescein diacetate (FDA) hydrolysis is a measurement of total microbial activity in soils and was determined in incubations using 60 mM potassium phosphate with pH 7.6 as a buffer and FDA stock solution (0.1%, w/v) as a substrate at 30 ºC for 20 min, stopping the reaction with chloroform/methanol (2:1 v/v) and measuring spectrophotometrically the absorbance at 490 nm (Adam and Duncan, 2001). Microbial C and N, and enzyme activities were measured with soil samples collected both in spring and autumn.

Table 4.1 Action of studied enzyme activities in soils.

Enzyme activity	Acronym	Enzyme commission	Action
β-glucosidase	BGL	EC 3.2.1.21	Hydrolyse hemicelluloses and cellulose mobilising C
Acid phosphatase	ACP	EC 3.1.3.2	Hydrolyses organic phosphates releasing inorganic P
N-acetyl-glucosaminidase	NAG	EC 3.2.1.30	Hydrolase chitin mobilising N
Leucyl-aminopeptidase	LAP	EC 3.4.11.1	Hydrolase proteins mobilising N
Dehydrogenase	DH	EC 1.x.	Oxidoreductases that oxidizes a substrate by reducing an electron acceptor
Fluorescein diacetate hydrolysis	FDA		FDA is hydrolysed by free and membrane bound enzymes (non-specific esterases, proteases and lipases)

In spring, multiple substrate-induced respiration (SIR) for different carbon sources was determined by the MicroResp^{TM} colorimetric method, to obtain Community-Level Physiological Profiles (CLPP) (Campbell et al., 2003). We measured basal respiration with water and substrate induced respiration for 11 different carbon sources (Table 4.2). We studied the mineralization of different forms of carbon with a gradient from labile to recalcitrant. Substrates were dissolved in deionized water and prepared as a stock solution at different concentrations (in mg of C g^{-1} of soil water) according to their water solubility (Table 4.2). Before and after 6 h of incubation at 25°C, detection plates were measured spectrophotometrically, recording the absorbance at 570 nm.

Table 4.2 Concentrations of different carbon sources added to soil for MicroResp™ assays.

Carbon source	Acronym	Concentration (mg C g^{-1} soil water)	Formula	Chemical group
Water	Water		H_2O	
N-Acetyl-D-glucosamine	AcG	7.5	$C_8H_{15}NO_6$	Carbohydrate
Alpha-Ketoglutaric acid	aKG	30	$C_5H_6O_5$	Carboxylic acid
L-Alanine	ALA	7.5	$C_3H_7NO_2$	Amino acid
Citric acid	CitA	30	$C_6H_8O_7$	Carboxylic acid
Gamma-Aminobutyric acid	GABA	30	$C_4H_9NO_2$	Amino acid
Alpha-D-Glucose	Glu	30	$C_6H_{12}O_6$	Carbohydrate
L-Malic acid	MalA	30	$C_4H_6O_5$	Carboxylic acid
Quercetin	Que	0.056	$C_{15}H_{10}O_7$	Flavonoid
Salicylic acid	SalA	2	$C_7H_6O_3$	Carboxylic acid
Trehalose	Tre	30	$C_{12}H_{22}O_{11}$	Carbohydrate
Vanillic acid	VanA	6	$C_8H_8O_4$	Phenolic acid

4.2.4. Data analysis

Differences in soil chemistry and CLPP between plant cover type, sampling site and their interactions were tested by two-way analysis of variance (ANOVA). In the case of microbial C and N, and enzyme activities we used repeated measures ANOVA to test differences between plant cover, site, season and their interactions. When effects were significant, the multiple comparison between means was tested by Tukey´s honest significant different (HSD) *post hoc*. Statistical significance threshold was fixed at $p \leq 0.05$. We tested normality with Shapiro–Wilk test, and homoscedasticity with Levene´s test. When ANOVA assumptions were not met, data was Box-Cox transformed or a constant variance function (*varIdent* function) was included in the ANOVA model. A non-parametric Scheirer–Ray–Hare test and a Dunn´s test corrected by Bonferroni *post hoc* were performed when data did not meet the assumptions.

Correlations between soil abiotic and biotic variables were evaluated by Pearson's correlation adjusted with Benjamini-Hochberg correction to control for "false discovery rate" derived from multiple testing.

The variation in soil catabolic functioning CLPP was represented in a non-metric multidimensional scaling (NMDS) ordination biplot, distinguishing plant cover and site factors by different symbols. Data matrix was transformed by Hellinger, and Euclidean distance was used as the measure of dissimilarity. We tested the effects of plant cover and site factors on catabolic functioning with a permutation multivariate analysis of variance (PERMANOVA).

The catabolic diversity of the soil microbial communities was quantified using a carbon substrate diversity index calculated with the Shannon index with the MicroResp™ data:

$$\text{Shannon Index} = -\sum_{i=1}^{s} p_i \ln p_i$$

In the Shannon index, *p* is the ratio of the CO_2 rate for a carbon source to the sum of CO_2 rates for all substrates, and *s* is the number of substrates.

Total trace element concentrations were compared with background values (BV) for the South Portuguese Zone (Galán and Romero, 2008). The reference level (percentile 90) was selected as this is the maximum value accepted for non-contaminated soils.

Statistical analyses were carried out using R software v. 3.6.1 (R development Core Team) using *ggplot2*, *nlme* and *vegan* (Oksanen et al., 2016) packages.

4.3. Results

4.3.1 Soil properties

Soil moisture varied across seasons (F = 1212; $p < 0.001$) with a severe decrease in autumn (Fig. 4.2). Significant differences were found within each season; in spring there was only a significant effect of plant cover (F = 3.78; $p = 0.016$), while in autumn there was a significant interaction between plant cover and site (F = 3.49; $p = 0.023$). In spring, white poplar soils had more water content than grassland soils at both sites. In autumn, at the South site white poplar and stone pine soils retained higher water content than grassland soils (Fig. 4.2).

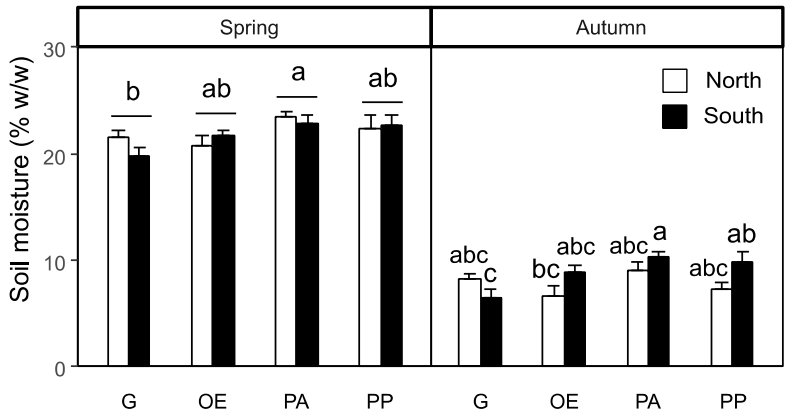


Figure 4.2 Soil moisture (%) according to plant cover (G = Grassland, OE = *Olea europaea*, PA = *Populus alba*, PP = *Pinus pinea*), site (North and South) and season (Autumn and Spring) factors. Letters indicate differences between plant cover (Spring) or site x plant cover interaction (Autumn) ($p \leq 0.05$).

Soil chemical properties were significantly different between plant cover and site, but no interaction was found (Table 4.3). The most significant differences were found in pH, being the North site significantly more acidic than the South site. In terms of plant cover, in the North site pine soils were significantly more acidic than grassland soils, while in the South site wild olive soils were significantly more acidic than grassland and pine soils. Total N was similar between sites and only in the South site N was significantly higher in wild olive soils compared to grassland soils. Total C presented a higher percentage in the South site, however organic C was similar between sites. Organic C was affected by plant cover only in the South site being significantly higer under pine and white poplar compared to grassland soils. The C:N ratio was significantly different between plant covers with a higher ratio in pine and white poplar soils compared to grassland and wild olive soils (Table 4.3). Available P was higher at the South site, and the studied total trace elements (As, Cr, Cu, Fe, Mn, Pb, S and Zn) were significantly higher at the North site. Total trace element concentrations at the North site were higher than soil background values of non-contaminated soils for As, Cu, Pb and Zn, while in the South site background values were exceeded only for Zn (Table 4.3).

Table 4.3 One-way ANOVA of plant cover (Grassland, *Olea europaea*, *Populus alba* and *Pinus pinea*) (*df* = 3) statistic F and two-way ANOVA of Site (North and South) (*df* = 1) and interaction (Site x Plant cover) (*df* = 3) on soil abiotic variables including total concentration of trace elements (n = 56). Background values (BV) at percentile 90 (P 90) in South Portuguese Zone soils at depth 0-20 cm in mg kg^{-1}, according to Galán and Romero (2008). Tukey's honest significant difference (HSD) *post hoc* with letters indicating differences among levels of the Plant cover factor within each site: the North site (lowercase letters) and the South site (uppercase letters). Asterisks indicate the significance level (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$). δ: Non-parametric Kruskal-Wallis/ Schneirer-Ray-Hare test and H statistic; ¥: Box-cox transformation; α: *VarIdent* function for homoscedasticity. Org. C: organic carbon. Av. P: available P.

	Site	Plant cover				BV (P 90)	F		
		Grassland	*Olea europaea*	*Populus alba*	*Pinus pinea*		Plant cover	Site	Interaction
pH	North	6.04±0.28**a**	5.62±0.30**ab**	5.22±0.25**ab**	4.64±0.40**b**		**3.64***	**40.66***** δ	4.38 δ
	South	7.23±0.01**A**	6.84±0.05**C**	6.99±0.01**BC**	7.17±0.04**AB**		**22.41***** δ		
Total N	North	0.232±0.019	0.250±0.013	0.313±0.035	0.231±0.023		2.56	1.28	1.19
(%)	South	0.210±0.015**B**	0.267±0.013**A**	0.258±0.013**AB**	0.227±0.015**AB**		**3.59***		
Total C	North	1.61±0.23	1.87±0.18	2.72±0.42	2.41±0.36		2.65	**13.26*****	0.60
(%)	South	2.34±0.26**A**	3.32±0.24**A**	3.46±0.26**A**	3.39±0.40**A**		**3.10***		
Org. C	North	1.52±0.23	1.81±0.18	2.67±0.42	2.33±0.36		2.72	1.37	0.57
(%)	South	1.13±0.19**B**	1.98±0.18**AB**	2.15±0.25**A**	2.14±0.35**A**		**3.78***		
C:N	North	6.33±0.48**b**	7.16±0.47**b**	8.30±0.39**ab**	9.77±0.83**a**		**6.84****	0.99	0.41
	South	5.15±0.70**B**	7.29±0.43**AB**	8.18±0.67**A**	9.13±0.93**A**		**5.81****		

Av. P	North	8.43±2.42	7.54±1.05	11.70±1.90	8.83±1.23		1.78 ¥	**33.84*** **	1.98
(mg kg^{-1})	South	17.20±2.94	21.99±2.82	18.13±2.33	13.76±1.12		1.97		
As	North	132.3±18.4	121.0±13.9	152.3±29.3	143.4±13.0	157	0.60 α	**243.05*** ** ¥ α	0.92 ¥ α
(mg kg^{-1})	South	43.6±6.6	42.5±8.4	38.8±5.6	34.0±2.2		0.27 ¥		
Cr	North	45.63±5.51	49.79±7.25	34.70±4.63	41.18±2.19	209	1.47	**8.80** ¥	1.74 ¥
(mg kg^{-1})	South	32.70±4.13	31.20±2.13	33.06±1.20	34.38±3.08		0.29 α		
Cu	North	189.2±11.8	182.8±11.4	173.8±19.1	155.8±8.9	108	1.19	**382.95*** ** α	0.99 α
(mg kg^{-1})	South	53.5±7.2	50.6±5.0	47.4±4.9	45.5±5.0		0.41		
Fe	North	47583±2602	44297±2125	44644±3154	41584±1661		1.00	**257.52*** **	0.88
(mg kg^{-1})	South	22253±1457	23052±1207	21806±1423	22396±944		0.164		
Mn	North	634±50	638±45	602±48	508±47		1.60	**97.95*** ** α	1.52 α
(mg kg^{-1})	South	348±23	358±22	315±22	346±14		0.81		
Pb	North	235.2±35.5	204.2±31.3	249.8±44.9	220.2±18.8	117	0.33	**102.75*** ** α	0.28 α
(mg kg^{-1})	South	86.8±12.4	83.8±17.2	78.5±10.0	70.9±5.1		0.20 ¥		
S	North	2585±609	2607±413	5951±1728	5248±1036		2.33 ¥	**122.27*** ** ¥	2.35 ¥
(mg kg^{-1})	South	1139±116	1013±132	873±91	915±67		1.36 ¥		
Zn	North	310.5±35.4**ab**	301.6±30.4**ab**	374.0±21.5**a**	267.3±31.0**b**	134	**3.03*** ¥	**17.17*** ** ¥	0.86 ¥
(mg kg^{-1})	South	257.9±31.6	234.6±13.0	243.1±25.7	219.5±14.5		0.31 ¥		

4.3.2 Soil microbial C and N

Soil microbial C and N followed a similar trend, showing significant differences between site, season and their interaction (Table 4.4). Both microbial C (Site x Season: F = 37.89, $p < 0.001$) and N (Site x Season: F = 7.45, $p = 0.008$) were significantly higher at the South site in autumn (Table 4.4). Plant cover effect was significant in spring (Table 4.4), when microbial C and N were reduced in grassland soils compared to white poplar (Fig. 4.3). Microbial biomass C:N ratio only showed significant differences in the interaction between site and season (F = 6.23, $p = 0.014$); in autumn this ratio was higher at the South site (Table 4.4; Fig. 4.3).

Table 4.4 Repeated measures ANOVA on soil microbial C, N and ratio (n = 56). Statistic F and *p*-values indicate effects of site (North and South) (*df* = 1), plant cover (Grassland, *Olea europaea*, *Populus alba* and *Pinus pinea*) (*df* = 3), season (Aut = Autumn and Spr = Spring) (*df* = 1) and interactions. Significant *p*-values ($p \leq 0.05$) are in bold. Two-way ANOVA effects of plant cover and site within each season. Asterisks indicate the significance level (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; - non-significant) within each season.

	Microbial C				**Microbial N**				**Microbial C:N**			
	F	*p*	*Aut*	*Spr*	F	*p*	*Aut*	*Spr*	F	*p*	*Aut*	*Spr*
Plant cover	2.11	0.104	-	**	1.84	0.146	-	**	1.03	0.384	-	-
Site	46.14	**<0.001**	***	-	18.79	**<0.001**	***	*	2.10	0.151	*	-
Plant cover x Site	2.65	0.053	*	-	1.12	0.346	-	-	0.54	0.657	-	-
Season	19.26	**<0.001**			9.22	**0.003**			2.60	0.110		
Site x Season	37.89	**<0.001**			7.45	**0.008**			6.23	**0.014**		
Plant cover x Site x Season	2.33	0.079			0.82	0.488			0.80	0.498		

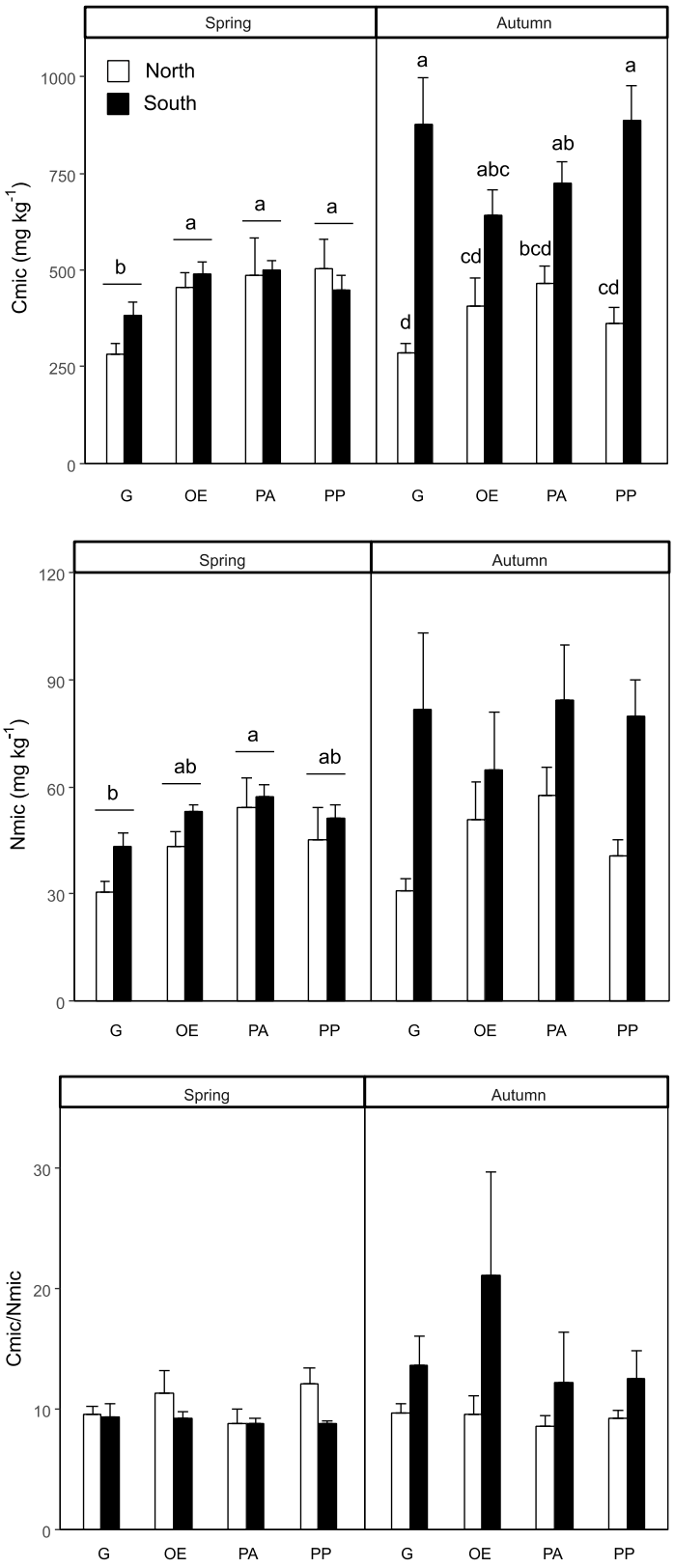


Figure 4.3 Two-way ANOVA effects of plant cover (G = Grassland, OE = *Olea europaea*, PA = *Populus alba*, PP = *Pinus pinea*) and site (North and South) within each season. Letters indicate differences of site x plant cover interaction or differences in plant cover when interaction was not significant ($p \leq 0.05$). Cmic: microbial C, Nmic: microbial N.

4.3.3 Soil enzyme activities

For all measured enzyme activities there was a significant triple interaction between plant cover, site and season factors (Table 4.5). Dehydrogenase activity was higher in spring at the South site and in autumn at the North site. In both seasons, DH activity was reduced in stone pine soils, especially at the North site (Fig. 4.4). Fluorescein diacetate hydrolytic activity was significantly higher at the North site in spring, but a site and plant cover interaction was found in autumn. White poplar soils at the North site and stone pine and grassland soils at the South site had significantly higher FDA hydrolytic activity than wild olive and white poplar at the South site (Fig. 4.4).

Significant differences were found in LAP activity for all factors and their interactions (Table 4.5). Over three times more LAP activity was measured in autumn compared to spring. At the South site, white poplar and stone pine soils had more LAP activity compared to wild olive and grassland, while at the North site LAP activity was reduced under stone pine and grassland soils (Fig. 4.4).

Beta glucosidase and ACP activities were significantly different between plant cover, site and season factors and their interactions, except plant cover and site interaction (Table 4.5). Beta glucosidase activity was higher under white poplar for both seasons compared to grasslands (in spring) and wild olive (in autumn). Moreover, in spring at the North site, soils presented a higher BGL activity than at the South site (Fig. 4.4). Acid phosphatase activity showed different trends within each season. In autumn, ACP highest activity was measured in grassland soil at the North site, however at the South site ACP activity was reduced in grassland and wild olive soils in comparison to the high activity found under stone pine. In spring, ACP activity at the North site was higher than at the South site, and stone pine ACP activity was higher in comparison to grassland soils (Fig. 4.4). N-acetyl-glucosaminidase activity was not significantly different between studied seasons or affected by the interaction between plant cover and site (Table 4.5). Enzyme activities with the same optimal acidic pH (BGL, ACP and NAG) presented similar trends; in spring, soils at the North site had higher BGL, ACP and NAG activities than those at the South site. In autumn, these differences between sites were reduced and the lowest BGL, ACP and NAG activities were registered under wild olive trees.

Table 4.5 Repeated measures ANOVA on dehydrogenase (DH), fluorescein diacetate (FDA), leucyl aminopeptidase (LAP), beta glucosidase (BGL), acid phosphatase (ACP) and N-acetyl-glucosaminidase (NAG) activities (n = 56). Statistic F and *p*-values indicate effects of site (North and South) (*df* = 1), plant cover (Grassland, *Olea europaea*, *Populus alba* and *Pinus pinea*) (*df* = 3), season (Aut = Autumn and Spr = Spring) (*df* = 1) and interactions. Significant *p*-values ($p \leq 0.05$) are in bold. Two-way ANOVA effects of plant cover and site within each season. Asterisks indicate the significance level (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; - non-significant) within each season.

	DH				**FDA**				**LAP**			
	F	*p*	*Aut*	*Spr*	F	*p*	*Aut*	*Spr*	F	*p*	*Aut*	*Spr*
Plant cover	8.89	**<0.001**	*	***	5.00	**0.003**	***	-	3.17	**0.028**	-	**
Site	1.50	0.223	***	***	68.26	**<0.001**	*	***	91.00	**<0.001**	***	***
Plant cover x Site	1.94	0.128	-	-	4.03	**0.010**	***	-	9.68	**<0.001**	***	***
Season	0.09	0.765			3.85	0.053			3780	**<0.001**		
Site x Season	65.90	**<0.001**			28.73	**<0.001**			54.53	**<0.001**		
Plant cover x Site x Season	3.43	**0.020**			3.23	**0.026**			3.85	**0.012**		

	BGL				**ACP**				**NAG**			
	F	*p*	*Aut*	*Spr*	F	*p*	*Aut*	*Spr*	F	*p*	*Aut*	*Spr*
Plant cover	5.35	**0.002**	**	**	6.72	**<0.001**	***	-	10.52	**<0.001**	***	**
Site	9.41	**0.003**	-	***	95.86	**<0.001**	-	***	36.53	**<0.001**	-	***
Plant cover x Site	1.32	0.273	-	-	2.13	0.102	***	*	0.42	0.596	-	-
Season	35.63	**<0.001**			8.28	**0.005**			0.28	0.737		
Site x Season	22.66	**<0.001**			64.11	**<0.001**			41.86	**<0.001**		
Plant cover x Site x Season	2.82	**0.043**			14.46	**<0.001**			2.73	**0.048**		

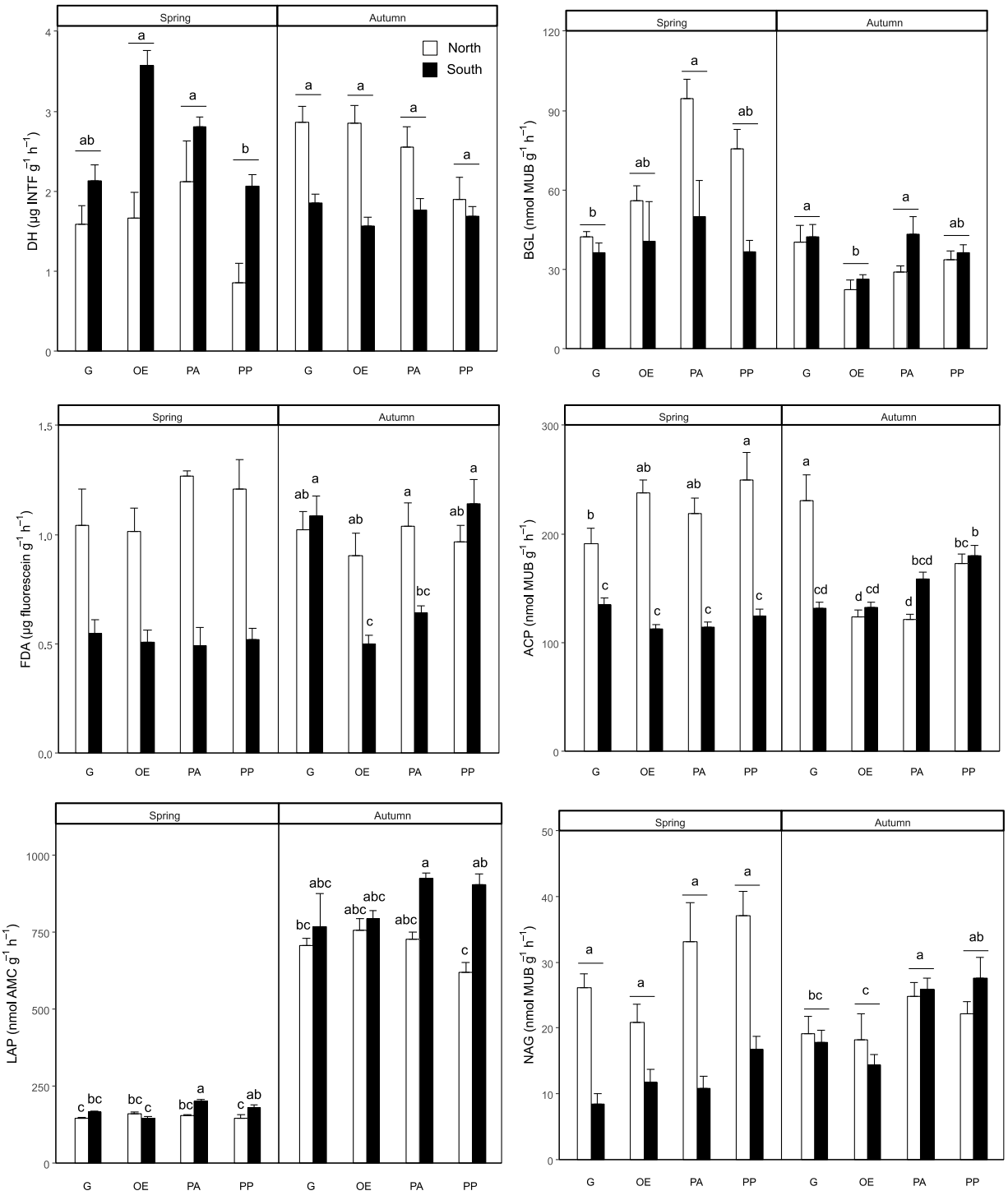


Figure 4.4 Two-way ANOVA effects of plant cover (G = Grassland, OE = *Olea europaea*, PA = *Populus alba*, PP = *Pinus pinea*) and site (North and South) within each season. Letters indicate differences of site x plant cover interaction or differences in plant cover (withn each season) when interaction was not significant ($p \leq 0.05$). DH: dehydrogenase, FDA: fluorescein diacetate incorporation, LAP: leucyl aminopeptidase, BGL: beta glucosidase, ACP: acid phosphatase, NAG: N-acetyl-glucosaminidase.

4.3.4 Correlations between soil biotic and abiotic variables

Correlations between soil variables were highly different between the two studied seasons; in spring the number of significant correlations was much higher than in autumn for all the biotic variables, except for microbial C biomass and LAP (Table 4.6). In spring, all enzyme activities were highly correlated with soil pH. FDA, BGL, ACP and NAG presented higher activities in acidic soils, while LAP and DH activities showed a positive correlation to soil pH. The correlation between enzyme activities and total trace elements or pH had opposite directions, except for Cr which did not correlate to any enzyme activity. Total N and organic C was positively correlated with BGL and NAG activities, but total C was negatively correlated with FDA and ACP activities. Available P presented a positive correlation to DH but a negative one to FDA, ACP and NAG activities. Also in spring, a positive correlation was found between microbial N biomass and soil pH, moisture, C, N and P, while a negative correlation was found with some total trace elements (As, Cu, Fe, Pb and S). Microbial C and C:N ratio only correlated to a few soil abiotic variables; C correlated to P (positively) and two trace elements, As and S (negative) while C:N ratio correlated to moisture and soil pH (negatively) (Table 4.6).

In contrast to the spring season, in autumn, abiotic variables exerted a limited influence on soil enzyme activities. No correlations were found between any soil chemical property and microbial C:N ratio, nor with the activities FDA, BGL, ACP or NAG. Soil pH did not correlate to any biotic variable, showing a variation in the influence of soil pH on microbial activity from spring to autumn season. Correlations between the indexes of microbial activity and total trace elements concentrations were also reduced in autumn. Microbial C and LAP activities, which were higher in the autumn season, did maintain the maximum number of correlations in this season. Microbial C showed a positive correlation to moisture, total C and P, but a negative correlation to As, Fe and Mn. LAP activity presented a positive correlation to moisture and P, but a negative correlation to total N, organic C and total trace elements (As, Cu, Pb and S). Dehydrogenase activity showed a different tendency between seasons; being positively correlated to Fe in autumn while negatively in spring. However, microbial N followed a similar tendency in both seasons with a correlation with soil P (positive correlation) and Fe (negative correlation) (Table 4.6).

Table 4.6 Results of the bivariate Pearson´s correlation coefficients between soil biotic and abiotic variables in spring and autumn. Significant *p*-values ($p \leq 0.05$) are in bold. Asterisks indicate the significance level (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$). Cmic: biomass carbon; Nmic: biomass nitrogen; C:Nmic: biomass C:N ratio; DH: dehydrogenase; FDA: fluorescein diacetate; LAP: leucyl aminopeptidase; BGL: beta-glucosidase; ACP: acid phosphatase and NAG: N-acetyl-glucosaminidase.

Spring

	Cmic	Nmic	C:Nmic	DH	FDA	LAP	BGL	ACP	NAG
Moisture	0.224	**0.435****	**-0.385****	0.026	0.089	0.163	0.082	-0.060	0.232
pH	0.136	**0.379***	**-0.375***	**0.659*****	**-0.622*****	**0.490*****	**-0.502*****	**-0.876*****	**-0.673*****
Total N	0.196	**0.375***	-0.247	0.046	0.168	-0.017	**0.304***	0.080	**0.353***
Total C	0.294	**0.465*****	-0.226	0.254	**-0.313***	0.264	-0.024	**-0.386****	-0.055
Org. C	0.252	**0.331***	-0.094	-0.112	0.192	-0.022	**0.324***	0.171	**0.449****
C:N	0.264	0.236	0.050	-0.179	0.175	-0.006	0.233	0.168	**0.376***
Av. P	**0.337***	**0.485*****	-0.203	**0.608*****	**-0.385****	0.265	-0.238	**-0.668*****	**-0.474*****
As	**-0.316***	**-0.396****	0.089	**-0.621*****	**0.509*****	**-0.429****	**0.462*****	**0.766*****	**0.726*****
Cr	-0.068	-0.119	0.058	-0.086	0.208	-0.063	0.026	0.252	0.115
Cu	-0.169	**-0.318***	0.181	**-0.548*****	**0.641*****	**-0.404****	**0.366***	**0.768*****	**0.616*****
Fe	-0.227	**-0.35***	0.141	**-0.539*****	**0.601*****	**-0.411****	**0.386****	**0.749*****	**0.656*****
Mn	0.066	-0.012	0.077	-0.175	**0.585*****	-0.296	0.296	**0.519*****	**0.469*****
Pb	-0.292	**-0.352***	0.050	**-0.547*****	**0.453****	**-0.375***	**0.428****	**0.674*****	**0.670*****
S	**-0.309***	**-0.349***	0.060	**-0.595*****	**0.418****	**-0.385****	**0.523*****	**0.684*****	**0.711*****
Zn	0.137	0.164	-0.094	-0.063	**0.340***	-0.122	**0.308***	0.252	**0.342***

Autumn

	Cmic	Nmic	C:Nmic	DH	FDA	LAP	BGL	ACP	NAG
Moisture	**0.442****	0.243	0.124	-0.127	0.097	**0.484****	0.127	0.069	0.294
pH	0.348	0.107	0.179	-0.182	-0.120	-0.278	0.125	-0.180	-0.177
Total N	0.120	-0.053	0.101	-0.111	-0.087	**-0.568*****	-0.085	-0.137	-0.204
Total C	**0.525*****	0.299	0.153	**-0.519*****	-0.166	0.009	0.050	-0.191	-0.002
Org. C	0.134	-0.042	0.083	-0.151	-0.075	**-0.565*****	-0.101	-0.120	-0.170
C:N	0.050	0.071	-0.097	-0.211	0.046	0.157	-0.116	0.075	0.222
Av. P	**0.652*****	**0.458****	0.260	**-0.376***	-0.131	**0.636*****	0.098	-0.258	-0.123
As	**-0.525*****	-0.326	-0.186	0.269	0.187	**-0.408***	-0.118	0.110	0.027
Cr	-0.299	-0.202	-0.122	0.306	-0.027	-0.196	-0.033	0.243	-0.227
Cu	0.091	-0.089	0.098	-0.082	-0.070	**-0.597*****	-0.115	-0.094	-0.215
Fe	**-0.598*****	**-0.359***	-0.233	**0.470****	0.242	-0.279	-0.115	0.213	0.036
Mn	**-0.554*****	-0.322	-0.199	**0.682*****	0.187	-0.149	-0.126	0.102	-0.042
Pb	-0.280	-0.304	-0.027	0.121	0.071	**-0.728*****	-0.093	0.013	-0.180
S	-0.331	-0.139	-0.183	-0.005	0.144	**-0.359***	-0.122	-0.037	0.121
Zn	-0.267	-0.203	-0.071	**0.416***	0.176	0.011	0.052	-0.105	0.001

4.3.5 Correlations between soil biotic variables

The highest positive correlation was between microbial C and N biomass (Fig. 4.5). LAP was the only enzyme activity that positively correlated with microbial C and N, while ACP negatively correlated to microbial N. FDA and DH correlated with ACP and NAG activities although in opposite directions; a positive correlation with FDA and a negative correlation with DH. The activities of BGL, ACP and NAG showed a strong positive correlation between them and BGL-LAP correlation was significantly negative (Fig. 4.5).

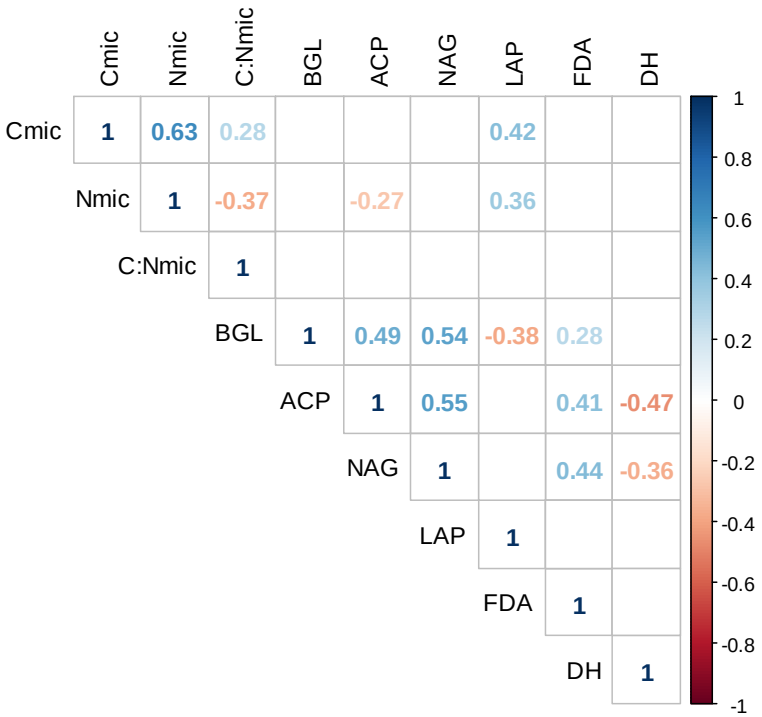


Figure 4.5 Results of the bivariate Pearson´s correlation coefficients between soil abiotic variables (joint data from autumn and spring). Blank cells have no significant coefficient ($p < 0.05$). Cmic: microbial C; Nmic: microbial N; BGL: beta-glucosidase; ACP: acid phosphatase and NAG: N-acetyl-glucosaminidase; LAP: leucyl aminopeptidase; FDA: fluorescein diacetate; DH: dehydrogenase.

4.3.6 Soil catabolic diversity

There were significant differences in the utilization of acetyl glucosamine, salicylic acid and trehalose among plant covers (Table 4.7). Utilization of these three carbon sources increased with tree afforestation, in particular under white poplar cover, followed by stone

pine cover. The only significant difference found between sites was the utilization of aminobutyric acid, which was higher in the North site soils. No interaction was found between plant cover and site factors. Functional diversity index (Shannon) was not significantly influenced by the studied factors (Table 4.7).

Table 4.7 Two-way ANOVA statistic F with effects of plant cover (Grassland, *Olea europaea*, *Populus alba* and *Pinus pinea*) (*df* = 3), site (North and South) (*df* = 1), and their interaction (Int.) (*df* = 3) of the catabolic profile of studied soils with 12 carbon substrates and Shannon index using MicroResp™ method, expressed in µg CO_2-C g^{-1} h^{-1}. Tukey's HSD *post hoc* with letters indicating differences among plant cover levels. Asterisks indicate the significance level (* $p \leq 0.05$; ** $p \leq 0.01$). Substrates acronyms: basal respiration (Water), acetyl glucosamine (AcG), α-Ketoglutaric acid (aKG), L-Alanine (ALA), citric acid (CitA), aminobutyric acid (GABA), α-D-Glucose (Glu), L-Malic acid (MalA), quercetin (Que), salicylic acid (SalA), trehalose (Tre) and vanillic acid (VanA).

	Site	Plant cover				F		
		Grassland	*Olea europaea*	*Populus alba*	*Pinus pinea*	Plant cover	Site	Int.
Water	North	2.56±0.42	2.91±0.83	4.39±0.56	3.26±1.23	1.42	0.12	0.18
	South	3.26±0.55	3.25±0.83	4.15±0.49	3.16±0.56			
AcG	North	6.63±0.61**B**	6.66±1.05**AB**	9.55±1.02**A**	7.33±1.88**AB**	**3.15***	0.43	0.21
	South	5.71±0.92	6.22±0.75	8.54±0.61	7.78±0.89			
aKG	North	9.70±1.10	9.90±1.44	11.00±1.99	8.84±2.14	0.21	0.01	0.26
	South	9.95±1.47	9.10±0.81	9.94±0.58	10.10±1.39			
ALA	North	4.66±0.36	5.15±0.96	6.93±0.62	5.34±1.48	2.18	0.53	0.03
	South	5.11±0.86	5.61±0.65	7.12±0.79	6.05±0.85			
CitA	North	12.30±3.83	14.10±3.97	17.40±5.71	12.20±5.43	0.78	3.05	0.3
	South	14.70±2.47	32.10±4.64	20.00±2.03	18.60±3.14			
GABA	North	3.68±0.56	3.66±0.51	4.70±0.62	4.18±0.74	2.18	**4.93***	0.28
	South	2.56±0.51	3.26±0.42	4.12±0.21	3.00±0.47			
Glu	North	8.57±0.82	9.53±1.10	10.40±0.9	8.60±1.30	1.05	1.27	0.15
	South	9.24±0.39	10.70±0.86	10.50±0.57	9.88±1.63			
MalA	North	7.86±1.30	8.41±1.07	11.30±1.92	9.31±2.13	0.84	2.18	0.41
	South	9.83±0.86	10.90±1.56	10.90±0.85	11.40±1.57			
Que	North	1.93±0.23	3.10±1.31	4.54±0.87	2.85±0.65	2.09	1.58	0.91
	South	3.97±0.62	2.46±0.67	4.92±0.8	4.14±1.24			
SalA	North	4.64±0.54**B**	5.51±1.29**B**	8.30±0.70**A**	6.44±1.92**AB**	**4.02***	1.89	0.03
	South	3.99±0.84	4.35±0.81	7.09±0.74	5.41±0.78			

Tre	North	6.01±0.30**B**	8.07±1.08**B**	10.40±1.10**A**	8.77±1.75**AB**	**4.79****	0.01	0.26
	South	6.85±1.17	6.94±0.92	10.50±0.67	8.68±1.36			
VanA	North	2.78±0.19	3.20±0.70	4.23±0.43	3.56±0.77	1.81	0.01	0.04
	South	3.01±0.75	3.35±0.62	4.12±0.28	3.49±0.49			
Shannon	North	2.32±0.05	2.31±0.07	2.37±0.04	2.36±0.03	1.65	2.68	0.32
	South	2.29±0.05	2.22±0.06	2.36±0.01	2.30±0.03			

The influence of plant cover and site factors on catabolic diversity was tested with PERMANOVA, and the site factor seemed to influence the catabolic diversity of the soils ($F = 3.29$; $p = 0.035$; $R^2 = 0.057$). Plant cover ($F = 1.83$; $p = 0.084$; $R^2 = 0.095$) and plant cover and site factors interaction ($F = 0.31$; $p = 0.970$; $R^2 = 0.016$) effects were not significant. The South site was characterized by a higher use of citric acid as carbon source. At the North site a higher aminobutyric acid utilization was observed. There was a grouping effect of white poplar soils presenting the highest utilization of half of the carbon sources, including those of higher chemical complexity and lower biodegradability (acetyl glucosamine, L-Alanine, quercetin, salicylic acid, trehalose and vanillic acid) as well as showing the highest basal respiration (Fig. 4.6).

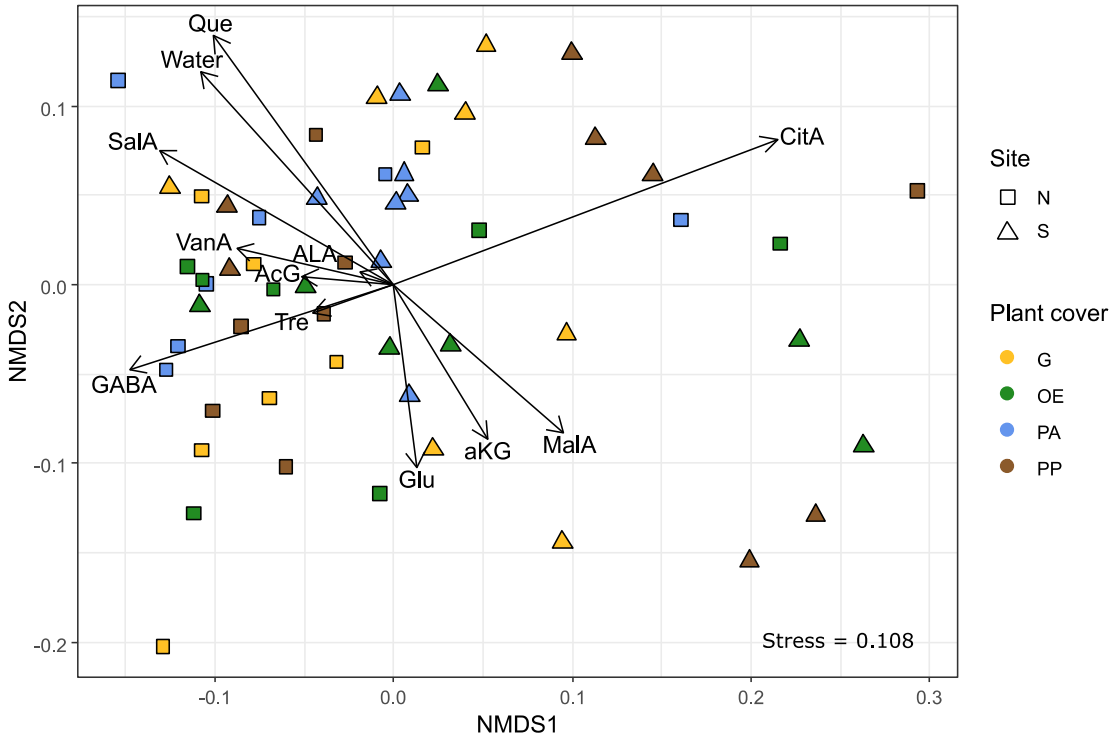


Figure 4.6 NMDS ordination plot of soil catabolic diversity according to plant cover and site factors. Symbols and colors according to site (N = North (square) and S = South (triangle)) and plant cover (G = Grassland, OE = *Olea europaea*, PA = *Populus alba*, PP = *Pinus pinea*).

Soil moisture correlated with most of the substrates assesed in respiration, showing a positive correlation (Fig. 4.7). Available P showed a positive correlation with citric acid and α-D-Glucose, while pH showed a positive correlation with citric acid and malic acid. Total N, organic C and Zn showed a postiive correlation with acetyl glucosamine. Contamination (As and S) presented a nehative correlation with citric acid. No significant correlations were found for α-Ketoglutaric acid substrate and the Shannon index (Fig. 4.7).

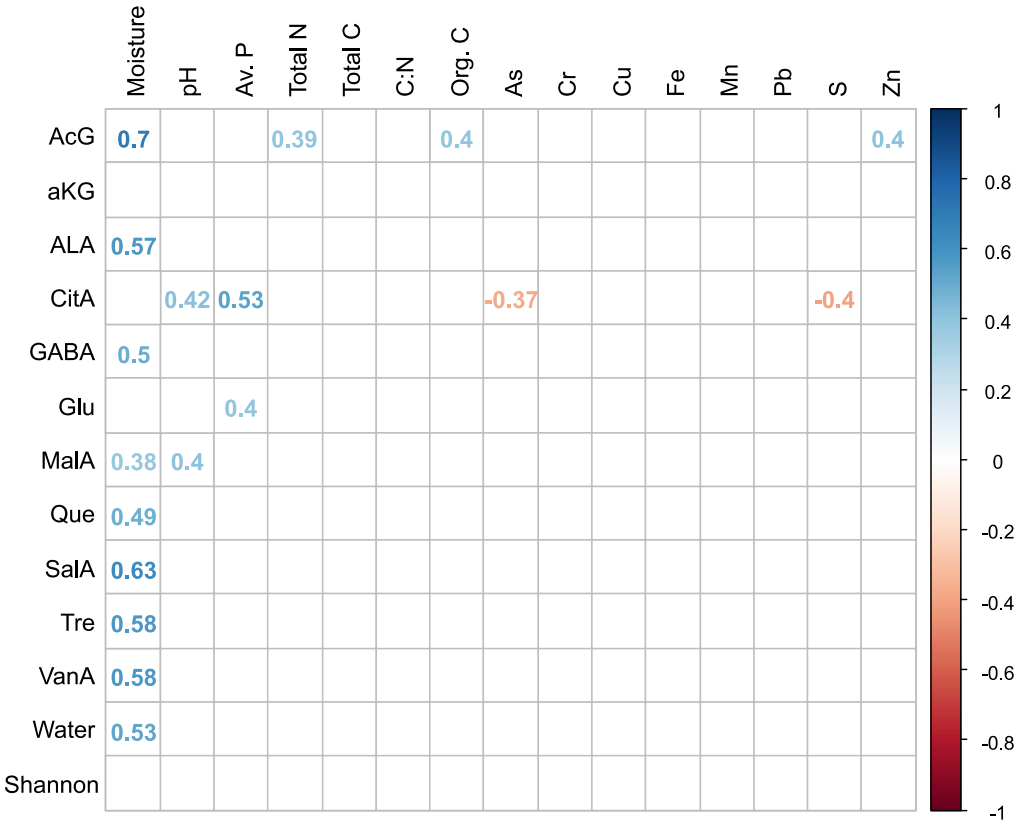


Figure 4.7 Results of the bivariate Pearson´s correlation coefficients between catabolic diversity and soil abiotic variables in spring. Blank cells have no significant coefficient ($p < 0.05$). Substrates acronyms: acetyl glucosamine (AcG), α-Ketoglutaric acid (aKG), L-Alanine (ALA), citric acid (CitA), aminobutyric acid (GABA), α-D-Glucose (Glu), L-Malic acid (MalA), quercetin (Que), salicylic acid (SalA), trehalose (Tre), vanillic acid (VanA) and basal respiration (Water).

4.4 Discussion

Soil biological parameters may have a potential as early and sensitive indicators of soil ecological stress and restoration effects (Dick and Tabatabai, 1992). In this study, we determined the effect of phytoremediation in the microbial activity of soils in a trace element contaminated area, and we aimed to disentangle the influence of different factors

such as tree identity, seasonality and soil abiotic properties on soil microbial functioning. Phytoremediation is one of the most cost-efficient strategies for *in situ* remediation of trace element contaminated soils, that promotes positive changes in the soil abiotic environment and the recovery of soil microbial community functionality (Pérez-De-Mora et al., 2006). Understanding tree afforestation effects on the recovery of soil multifunctionality in a degraded soil may provide a better knowledge of the functioning of soil microbial communities, and may improve future decisions about restoration management. We support here the importance of studying microbial activities as soil quality indices and to evaluate phytoremediation success (Burns et al., 2013; Wang et al., 2007).

4.4.1. Afforested tree species improve soil fertility and microbial biomass

Soil trace element contamination in the study area is still above background levels, 19 years after a phytoremediation strategy was implemented along the Guadiamar Green Corridor. The proximity to the mine tailings dam at the North site has resulted in lower pH and a higher concentration of total trace elements and, therefore, a higher ecological risk at this site. In a previous study, Domínguez et al. (2016) found strong soil acidification due to the oxidation of the remnants of sulphides deposited during the mine-spill, at the most contaminated areas in the Guadiamar Green Corridor. Therefore, mine-spill contamination may be responsible for the acidification effect in this site, enchanced by the low carbonate content of these soils that limit buffering capacity against changes in pH.

A marked species-specific effect through a chemical footprint on topsoil was found. Trees act as ecosystem engineers modifying the soil properties (Jones et al., 1994; Mitchell et al., 2010b), potentially through their root exudates and through leaf litter quantity and quality, besides modifying temperature and moisture. Subsequently, the decomposition of roots and leaf litter can produce an effect on some ecological processes such as nutrient and carbon cycling, contributing to the maintenance of soil fertility (Kara et al., 2014). Low-quality litter is generally indicated by high values of C:N ratio of the litter (Chomel et al., 2016). In our study, we found a species-specific chemical footprint in the soil, based on the data of the C:N of litter and soil surface. In previous studies, coniferous species

were found to produce lower quality litter compared to broadleaf species (Chomel et al., 2015; Cornwell et al., 2008; Pérez-Harguindeguy et al., 2000) and our study corroborated this litter effect as soils under the stone pine were those with the highest soil C:N ratios. Acidification of soil underneath pines trees, where litter with a high C:N accumulates, has also been often reported (Orozco-Aceves et al., 2015; Sariyildiz et al., 2005). This acidification effect was expected to be stronger in the naturally acidic soils, which are poor in carbonate and therefore with a lower capacity to buffer against pH changes. Our study confirms previous evidence of an acidifying effect of the plantation of pines at the acidic North site, which results in a higher availability of trace elements in the soils underneath pine species (Madejón et al., 2018). In addition, our study supports that soil trace element contamination and low pH reduces microbial biomass (Brookes, 1995; Wang et al., 2007).

Grassland soils, covered by herbaceous species, presented the lowest soil C:N ratio in comparison to soils underneath tree species, which is in line with the general understanding that soil of herbaceous habitats has lower-recalcitrant compounds in comparison to forested ones (Strickland et al., 2009). Moreover, herbaceous species are known to produce lower litter quantities and accumulation than trees (Donath and Eckstein, 2008) which could explain the low soil total C and N and organic C contents in grassland soils. The low C content explained why microbial and biochemical variables were lower in grasslands than in afforested soils; Moreno et al. (2009) found that the higher C content in forest enhanced microbial growth and organic matter bound-trace elements. The positive effect of tree afforestation on microbial biomass may be explained by their higher plant biomass, compared with grasslands, which may promote microbial growth due to higher nutrient availability and litter input.

4.4.2. Seasonal changes of microbial biomass and enzyme activities

Seasonal changes in soil temperature and moisture affected microbial C and N, and the activities of LAP, BGL and ACP enzymes (but not DH and NAG activities). Microbial C (in autumn) and N (in spring) correlated with soil moisture, however, most enzyme activities were not significantly correlated with soil moisture. This result was in line with the study by Zeglin et al. (2013), suggesting that soil moisture influences microbial

functions in different ways. In a meta-analysis carried out by Ren et al. (2018), soil microbial biomass decline was related to rainfall reductions. In our study, microbial C was enhanced by soil moisture in autumn (but not in spring); despite the increased aridity in autumn 2017, the boost of nutrients from leaf fall in this season may explain microbial C growth. At the South site, microbial biomass and their C:N ratio were enhanced in the drier soil conditions of autumn. An explanation of this increase may be caused by a seasonal shift in the microbial community composition towards a higher abundance of organisms with higher tolerance to low soil moisture (Zeglin et al., 2013). The unusual aridity in autumn 2017 may have increased the proportion of fungi over bacteria, as fungi are more tolerant to drought than bacteria. As fungi accumulate higher levels of intracellular C compared to N under water stress conditions (Schimel et al., 2007), the result could the higher C:N ratio found in these soils, in comparison to the wetter soils in spring (Aponte et al., 2014; Jensen et al., 2003; Wilkinson et al., 2002).

Soil total microbial activity has been determined by measuring two different metabolic processes. Fluorescein diacetate method determines the hydrolysing activity of microbial organisms in the soil involved in soil organic matter decomposition, while DH activity determines the oxidative activity of several intracellular enzymes that catalyses the transfer of H^+ and electrons between compounds (Nannipieri et al., 2017). Results between FDA and DH were opposite and there was a lack of correlation between them, as well as between these variables and other microbial indicators such as microbial biomass,as found in other studies (Stubberfield and Shaw, 1990). In general, the effects of trace elements on microbial activity are known to be wide and depend on many variables such as climate, soil pH, texture and organic matter (Moreno et al., 2009). There is a consensus that trace elements entail a reduction of total microbial activity due to the toxic effects of these elements (Pérez-De-Mora et al., 2006), however, our results were contradictory. Soil pH and trace element concentrations seemed to cause a different trajectory between FDA and DH methods. Microbial activity determined by FDA was enhanced in acidic and highly contaminated soils (those in the North site), but DH activity was at a maximum with neutral pH and low trace element concentration soils. Tokuda and Hayatsu (2002) also found a higher FDA activity in acidic soils. Therefore, these methods (FDA and DH) are not comparable to measure total microbial activity when evaluating soils with different soil pH. In addition, the seasonal changes in FDA and DH were contradictory: FDA was higher at the most acidic and polluted site (North) in spring, while DH activity was more intense in autumn. In spring, the acidic soil and, potentially,

the acidification effect of the stone pine seemed to produce an inhibitory effect of DH activity at the acidic site; however, in autumn, DH activity increased in acidic soils and was decoupled from soil pH.

Other measured activities, such as BGL, ACP and NAG, also presented this decoupling from chemical soil variables in autumn, while in spring they showed significant links to abiotic variables. Microbial communities may be constrained by the physiological or nutrient limitation stress of the extended drought (Zeglin et al., 2013). In spring, when climate conditions were similar to previous years, soil microbial activities seemed to be more deterministic and were driven by soil abiotic variables. However, in autumn, microbial communities faced unusual drier conditions that probably increased stochasticity, disconnecting microbial activity from the soil characteristics. Microbial dynamics might be affected by drought conditions via changes in water content or microbial activity or via changes in functional responses or shift of the microbial community composition (Fierer et al., 2003; Zeglin et al., 2013). It is possible that only specific taxa were active in autumn under drought conditions (Aanderud and Lennon, 2011), reducing the link between microbial activities and soil abiotic environment. In autumn, a reduction of microbial diversity may occur with only a few microorganisms adapted to extreme conditions, or a proliferation of fungi over bacteria, as discussed above for microbial biomass, as fungi are more tolerant to drought than bacteria (Aponte et al., 2014; Jensen et al., 2003; Wilkinson et al., 2002). An evaluation of the structure of the microbial community and the relative abundances of different microbial functional groups, by PLFA or metabarcoding analysis, would have contributed to explain these patterns.

Enzyme activities, which are linked to microbial metabolism and biogeochemical processes, catalyse the hydrolysis of assimilable products from environmental sources of C (BGL), N (NAG and LAP) and P (ACP) reflecting the efficiencies of microbial nutrient assimilation and growth (Sinsabaugh et al., 2009). Among enzyme activities, LAP was the only activity that had consistent trends in both seasons: a positive correlation with moisture, pH and P, and a negative correlation with total N, organic C and trace elements. Previous results in our study area revealed a N limitation in these soils (Domínguez et al., 2010b). Therefore, it is likely that N is one of the main limiting nutrients for microbial growth in these soils; this fact may have enhanced the production and secretation of enzymes involved in N mineralization over the production of enzymes involved in the mineralization of other nutrients. Indeed, an increase in the activity of specific

hydrolyzing enzymes in response to a decrease in the availability of specific nutrients in soils is commonly reported in literature (reviewed in Burns et al., 2013). In our case, the extreme aridity in autumn 2017 enhanced LAP activity over three times more than in spring, coinciding with a potential limitation of N in microbial biomass, as indicated by the high C:N ratio. This increase represented the highest enzyme variation between seasons. In addition, LAP was the only enzyme with positive correlation to microbial biomass with no correlations to other enzymes (except negatively with BGL). All these evidences suggest that N might be the most limiting nutrient for microbial growth in our soils, and that the dynamics of N adquisition by microbes is highly influence by soil moisture in these sites.

In spring, BGL, ACP and NAG activities presented a higher activity in acidic soils as these activities are optimal under an acidic pH (Tabatabai, 1982). The high trace element concentrations (above background values) influenced these activities in spring but not in autumn soil samples. Moreover, afforestation seemed beneficial for some of these enzymes, enhancing BGL and NAG which showed a positive correlation to total N and organic C. The activity of acid phosphatase was reduced in the soils with the highest P availability, as these soils presented neutral pH (Dick et al., 2000).

4.4.3. Soil and tree species effects on microbial community-level physiological profile

We assessed microbial functional diversity with the measurement of the microbial heterotrophic capacity. Short-term soil respiration responses to different C substrates with varied complexity were determined by the MicroResp™ method, as this provides more accurate results of *in situ* conditions than other SIR bioassays (Lalor et al., 2007). The CLPP have been previously determined to detect changes in soil functionality due to mining disturbances (Cookson et al., 2008; Lewis et al., 2010).

Tree afforestation contributed to the increment of all SIR measures, especially under white poplar. The higher soil moisture, total and organic C content underneath white poplar, associated to a higher SOM content, seemed to enhance microbial biomass growth and higher SIR rates for most of the substrates (Bérard et al., 2014). Energy-poor and recalcitrant compounds such as quercetin and vanillic acid presented the lowest

respiration rates, however the highest utilization of these complex substrates was found by the soil microbiota under white poplar trees. In contrast, the respiration rates of carboxylic acids and carbohydrates were the highest among substrate types, as these substrates contains more labile C (Banning et al., 2012), but differential responses were found among these substrates.

Soil moisture had a large effect in the soil microbial metabolic profiles, among all measured abiotic variables. Nutrient availability is related to soil moisture, which could explain the highest soil respiration found under white poplar trees when compared to other tree species or grassland (Wakelin et al., 2013). It is probable that the water limitation encountered in this Mediterranean area may explain the positive relationship between soil respiration and moisture, as in a previous study carried out in an arid environment (Zhou et al., 2012). Apart from soil moisture, it is known the high impact of soil pH on CLPP (Ben Sassi et al., 2012; Zhou et al., 2012). In our study, soil pH and trace element concentrations influenced the different catabolic profiles between sites. A positive effect of soil pH on the catabolism of carboxylic acids (malic acid and citric acid) suggests that these compounds were optimized at high pH; however, they did not relate to organic C which was expected to increase the respiration capacity for labile C substrates (Creamer et al., 2016; Grządziel et al., 2019).

4.5 Conclusion

Our study on soil microbial activity in trace element contaminated soils showed that the applied phytoremediation strategy, with the afforestation of different tree species, entailed changes in soil properties, mainly increased SOM quantity and quality, as well as soil moisture. In particular, these soil properties were highly influenced by the white poplar trees. Microbial enzyme activities were good indicators of a seasonal drought stress. In spring, microbial communities were deterministic, as they were driven by soil abiotic conditions such as pH, C, N or P. In contrast, during the dry autumn 2017, a scarce relation with soil properties suggested an increased stochasticity of the microbial processes. The exception was the aminopeptidase activity, which was linked to the availability of N in soil at both seasons and increased in the autumn coinciding with a higher limitation of N for microbes.

In relation to the residual contamination of the site, soil pH and trace element concentrations were highly related to total microbial activity. Some enzyme activities (BGL, ACP and NAG) were enhanced under acidic soil conditions and the results were site-dependent. For other indicators, such as FDA and DH activities, comparison between soils with different pH must be carefully interpreted.

Regarding the effect of the planted trees, the soil microbiota under white poplars presented the highest catabolic activity for all substrates, likely because these soils had a higher SOM content and moisture. These soils also showed the highest rates of utilization of complex C substrates. These results suggest that the plantation of this tree species in semiarid regions with trace element contaminated soils provides special benefits given its positive effects on SOM that promotes a more active microbial community, as indicated by the respiration induced by a range of C sources. This study contributes to a better knowledge of functioning of soil microbial communities and to improve the restoration management of contaminated lands.

4.6 Bibliography

Aanderud, Z.T., Lennon, J.T., 2011. Validation of heavy-water stable isotope probing for the characterization of rapidly responding soil bacteria. Applied and Environmental Microbiology 77, 4589–4596.

Abaye, D.A., Lawlor, K., Hirsch, P.R., Brookes, P.C., 2005. Changes in the microbial community of an arable soil caused by long-term metal contamination. European Journal of Soil Science 56, 93–102.

Adam, G., Duncan, H., 2001. Development of a sensitive and rapid method for the measurement of total microbial activity using fluorescein diacetate (FDA) in a range of soils. Soil Biology and Biochemistry 33, 943–951.

Ali, A., Guo, D., Mahar, A., Wang, P., Shen, F., Li, R., Zhang, Z., 2017. Mycoremediation of potentially toxic trace elements—a biological tool for soil cleanup: a review. Pedosphere 27, 205–222.

Angst, G., Mueller, K.E., Eissenstat, D.M., Trumbore, S., Freeman, K.H., Hobbie, S.E., Chorover, J., Oleksyn, J., Reich, P.B., Mueller, C.W., 2018. Soil organic carbon stability in forests: distinct effects of tree species identity and traits. Global Change Biology 25, 1–18.

Aponte, C., García, L. V., Marañón, T., 2013. Tree species effects on nutrient cycling and soil biota: a feedback mechanism favouring species coexistence. Forest Ecology and Management 309, 36–46.

Aponte, C., Matías, L., González-Rodríguez, V., Castro, J., García, L. V., Villar, R., Marañón, T., 2014. Soil nutrients and microbial biomass in three contrasting Mediterranean forests. Plant and Soil 380, 57–72.

Ayres, E., Steltzer, H., Berg, S., Wall, D.H., 2009. Soil biota accelerate decomposition in high-elevation forests by specializing in the breakdown of litter produced by the plant species above them. Journal of Ecology 97, 901–912.

Baldrian, P., 2014. Distribution of extracellular enzymes in soils: spatial heterogeneity and determining factors at various scales. Soil Science Society of America Journal 78, 11–18.

Banning, N.C., Lalor, B.M., Cookson, W.R., Grigg, A.H., Murphy, D. V., 2012. Analysis of soil microbial community level physiological profiles in native and post-mining rehabilitation forest: which substrates discriminate? Applied Soil Ecology 56, 27–34.

Barajas-Aceves, M., 2005. Comparison of different microbial biomass and activity measurement methods in metal-contaminated soils. Bioresource Technology 96, 1405–1414.

Ben Sassi, M. Ben, Dollinger, J., Renault, P., Tlili, A., Bérard, A., 2012. The FungiResp method: An application of the MicroResp™ method to assess fungi in microbial communities as soil biological indicators. Ecological Indicators 23, 482–490.

Bérard, A., Mazzia, C., Sappin-Didier, V., Capowiez, L., Capowiez, Y., 2014. Use of the MicroResp™ method to assess Pollution-Induced Community Tolerance in the context of metal soil contamination. Ecological Indicators 40, 27–33.

Berg, J., McClaugherty, C., 2014. Plant litter: decomposition, humus formation, carbon sequestration, 3rd ed. Springer Berlin Heidelberg.

Brookes, P.C., 1995. The use of microbial parameters in monitoring soil pollution by heavy metals. Biology and Fertility of Soils 19, 269–279.

Burns, R.G., DeForest, J.L., Marxsen, J., Sinsabaugh, R.L., Stromberger, M.E., Wallenstein, M.D., Weintraub, M.N., Zoppini, A., 2013. Soil enzymes in a changing environment: current knowledge and future directions. Soil Biology and Biochemistry 58, 216–234.

Campbell, C.D., Chapman, S.J., Cameron, C.M., Davidson, M.S., Potts, J.M., 2003. A rapid microtiter plate method to measure carbon dioxide evolved from carbon substrate amendments so as to determine the physiological profiles of soil microbial communities by using whole soil. Applied and Environmental Microbiology 69, 3593–3599.

Chander, K., Joergensen, R.G., 2001. Decomposition of ^{14}C glucose in two soils with different amounts of heavy metal contamination. Soil Biology and Biochemistry 33, 1811–1816.

Chomel, M., Guittonny-Larchevêque, M., DesRochers, A., Baldy, V., 2016. Effect of mixing herbaceous litter with tree litters on decomposition and N release in boreal plantations. Plant and Soil 398, 229–241.

Chomel, M., Guittonny-Larchevêque, M., DesRochers, A., Baldy, V., 2015. Home field advantage of litter decomposition in pure and mixed plantations under boreal climate. Ecosystems 18, 1014–1028.

Clarholm, M., Skyllberg, U., 2013. Translocation of metals by trees and fungi regulates pH, soil organic matter turnover and nitrogen availability in acidic forest soils. Soil Biology and Biochemistry 63, 142–153.

Cookson, W.R., O'Donnell, A.J., Grant, C.D., Grierson, P.F., Murphy, D. V., 2008. Impact of ecosystem management on microbial community level physiological profiles of postmining forest rehabilitation. Microbial Ecology 55, 321–332.

Cornwell, W.K., Cornelissen, J.H.C., Amatangelo, K., Dorrepaal, E., Eviner, V.T., Godoy, O., Hobbie, S.E., Hoorens, B., Kurokawa, H., Pérez-Harguindeguy, N., Quested, H.M., Santiago, L.S., Wardle, D.A., Wright, I.J., Aerts, R., Allison, S.D., Van Bodegom, P., Brovkin, V., Chatain, A., Callaghan, T. V., Díaz, S., Garnier, E., Gurvich, D.E., Kazakou, E., Klein, J.A., Read, J., Reich, P.B., Soudzilovskaia, N.A., Vaieretti, M.V., Westoby, M., 2008. Plant species traits are the predominant control on litter decomposition rates within biomes worldwide. Ecology Letters 11, 1065–1071.

Creamer, R.E., Stone, D., Berry, P., Kuiper, I., 2016. Measuring respiration profiles of soil microbial communities across Europe using MicroResp™ method. Applied Soil Ecology 97, 36–43.

Dick, W.A., Cheng, L., Wang, P., 2000. Soil acid and alkaline phosphatase activity as pH adjustment indicators. Soil Biology and Biochemistry 32, 1915–1919.

Dick, W.A.D., Tabatabai, M.A., 1992. Significance and potential uses of soil enzymes. Soil Microbial Ecology 95–127.

Domínguez, M.T., Alegre, J.M., Madejón, P., Madejón, E., Burgos, P., Cabrera, F., Marañón, T., Murillo, J.M., 2016. River banks and channels as hotspots of soil pollution after large-scale remediation of a river basin. Geoderma 261, 133–140.

Domínguez, M.T., Marañón, T., Murillo, J.M., Schulin, R., Robinson, B.H., 2010. Nutritional status of Mediterranean trees growing in a contaminated and remediated area. Water, Air, and Soil Pollution 205, 305–321.

Donath, T.W., Eckstein, R.L., 2008. Grass and oak litter exert different effects on seedling emergence of herbaceous perennials from grasslands and woodlands. Journal of Ecology 96, 272–280.

Fierer, N., Schimel, J.P., Holden, P.A., 2003. Influence of drying-rewetting frequency on soil bacterial community structure. Microbial Ecology 45, 63–71.

Galán, E., Romero, A., 2008. Contaminación de suelos por metales pesados, in: Sociedad Española de Mineralogía. pp. 48–60.

Garbisu, C., Hernández-Allica, J., Barrutia, O., Alkorta, I., Becerril, J.M., 2002. Phytoremediation: a technology using green plants to remove contaminants from polluted areas. Reviews on Environmental Health 17, 173–188.

German, D.P., Weintraub, M.N., Grandy, A.S., Lauber, C.L., Rinkes, Z.L., Allison, S.D., 2011. Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies. Soil Biology and Biochemistry 43, 1387–1397.

Giller, K.E., Witter, E., McGrath, S.P., 1998. Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. Soil Biology and Biochemistry 30, 1389–1414.

Gregorich, E.G., Wen, G., Voroney, R.P., Kachanoski, R.G., 1990. Calibration of a rapid direct chloroform extraction method for measuring soil. Soil Biology and Biochemistry 22, 1009–1011.

Grosso, F., Iovieno, P., Alfani, A., Nicola, F. De, 2018. Structure and activity of soil microbial communities in three Mediterranean forests. Applied Soil Ecology 130, 280–287.

Grządziel, Furtak, K., Gałązka, A., 2019. Community-level physiological profiles of microorganisms from different types of soil that are characteristic to Poland -a long-term microplot experiment. Sustainability 11, 1–17.

Jensen, K.D., Beier, C., Michelsen, A., Emmett, B.A., 2003. Effects of experimental drought on microbial processes in two temperate heathlands at contrasting water conditions. Applied Soil Ecology 24, 165–176.

Jones, C.G., Lawton, J.H., Shachak, M., 1994. Organisms as ecosystem engineers. Oikos 69, 373–386.

Kandeler, E., Tscherko, D., Bruce, K.D., Stemmer, M., Hobbs, P.J., Bardgett, R.D., Amelung, W., 2000. Structure and function of the soil microbial community in microhabitats of a heavy metal polluted soil. Biology and Fertility of Soils 32, 390–400.

Kara, O., Bolat, I., Cakıroglu, K., Senturk, M., 2014. Litter decomposition and microbial biomass in temperate forests in Northwestern Turkey. Journal of Soil Science and Plant Nutrition 14.

Krumins, J.A., Goodey, N.M., Gallagher, F., 2015. Plant-soil interactions in metal contaminated soils. Soil Biology and Biochemistry 80, 224–231.

Lalor, B.M., Cookson, W.R., Murphy, D. V., 2007. Comparison of two methods that assess soil community level physiological profiles in a forest ecosystem. Soil Biology and Biochemistry 39, 454–462.

Lewis, D.E., White, J.R., Wafula, D., Athar, R., Dickerson, T., Williams, H.N., Chauhan, A., 2010. Soil functional diversity analysis of a bauxite-mined restoration chronosequence. Microbial Ecology 59, 710–723.

Madejón, P., Domínguez, M.T., Gil-Martínez, M., Navarro-Fernández, C.M., Montiel-Rozas, M.M., Madejón, E., Murillo, J.M., Cabrera, F., Marañón, T., 2018. Evaluation of amendment addition and tree planting as measures to remediate contaminated soils: the Guadiamar case study (SW Spain). Catena 166, 34–43.

Madejón, P., Domínguez, M.T., Madejón, E., Cabrera, F., Marañón, T., Murillo, J.M., 2018. Soil-plant relationships and contamination by trace elements: a review of twenty years of experimentation and monitoring after the Aznalcóllar (SW Spain) mine accident. Science of the Total Environment 625, 50–63.

Marañón, T., Navarro-Fernández, C.M., Domínguez, M.T., Madejón, P., Murillo, J.M., 2015. How the soil chemical composition is affected by seven tree species planted at a contaminated and remediated site. Web Ecology 15, 45–48.

Marx, M.C., Wood, M., Jarvis, S.C., 2001. A microplate fluorimetric assay for the study of enzyme diversity in soils. Soil Biology and Biochemistry 33, 1633–1640.

Mendez, M.O., Maier, R.M., 2008. Phytostabilization of mine tailings in arid and semiarid environments - An emerging remediation technology. Environmental Health Perspectives 116, 278–283.

Mitchell, R.J., Campbell, C.D., Chapman, S.J., Cameron, C.M., 2010. The ecological engineering impact of a single tree species on the soil microbial community. Journal of Ecology 98, 50–61.

Moreno, J.L., Bastida, F., Ros, M., Hernández, T., García, C., 2009. Soil organic carbon buffers heavy metal contamination on semiarid soils: effects of different metal threshold levels on soil microbial activity. European Journal of Soil Biology 45, 220–228.

Nannipieri, P., Ascher, J., Ceccherini, M.T., Landi, L., Pietramellara, G., Renella, G., 2017. Microbial diversity and soil functions. European Journal of Soil Science 68, 12–26.

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., 2016. Vegan: Community Ecology Package.

Olsen, S.R., Cole, C. V., Watanabe, F.S., Dean, L.A., 1954. Estimation of available phosphorus in soils by extraction with sodium bicarbonate, U. S. Department of Agriculture. Washington, D.C.

Orozco-Aceves, M., Standish, R.J., Tibbett, M., 2015. Long-term conditioning of soil by plantation eucalypts and pines does not affect growth of the native jarrah tree. Forest Ecology and Management 338, 92–99.

Pérez-De-Mora, A., Burgos, P., Madejón, E., Cabrera, F., Jaeckel, P., Schloter, M., 2006. Microbial community structure and function in a soil contaminated by heavy metals: effects of plant growth and different amendments. Soil Biology and Biochemistry 38, 327–341.

Pérez-Harguindeguy, N., Díaz, S., Cornelissen, J.H.C., Vendramini, F., Cabido, M., Castellanos, A., 2000. Chemistry and toughness predict leaf litter decomposition rates over a wide spectrum of functional types and taxa in central Argentina. Plant and Soil 218, 21–30.

Pérez-Izquierdo, L., Saint-André, L., Santenoise, P., Buée, M., Rincón, A., 2018. Tree genotype and seasonal effects on soil properties and biogeochemical functioning in Mediterranean pine forests. European Journal of Soil Science 69, 1087–1097.

Prescott, C.E., Grayston, S.J., 2013. Tree species influence on microbial communities in litter and soil: current knowledge and research needs. Forest Ecology and Management 309, 19–27.

Quilchano, C., Marañón, T., 2002. Dehydrogenase activity in Mediterranean forest soils. Biology and Fertility of Soils 35, 102–107.

Ren, C., Chen, J., Lu, X., Doughty, R., Zhao, F., Zhong, Z., Han, X., Yang, G., Feng, Y., Ren, G., 2018. Responses of soil total microbial biomass and community compositions to rainfall reductions. Soil Biology and Biochemistry 116, 4–10.

Sariyildiz, T., Anderson, J.M., Kucuk, M., 2005. Effects of tree species and topography on soil chemistry, litter quality, and decomposition in Northeast Turkey. Soil Biology and Biochemistry 37, 1695–1706.

Schimel, J., Balser, T., Wallenstein, M.D., 2007. Microbial stress-response physiology and its implications for ecosystem function. Ecology 88, 1386–1394.

Sinsabaugh, R.L., Hill, B.H., Follstad Shah, J.J., 2009. Ecoenzymatic stoichiometry of microbial organic nutrient acquisition in soil and sediment. Nature 462, 795–798.

Sinsabaugh, R.L., Lauber, C.L., Weintraub, M.N., Ahmed, B., Allison, S.D., Crenshaw, C., Contosta, A.R., Cusack, D., Frey, S., Gallo, M.E., Gartner, T.B., Hobbie, S.E., Holland, K., Keeler, B.L., Powers, J.S., Stursova, M., Takacs-Vesbach, C., Waldrop, M.P., Wallenstein, M.D., Zak, D.R., Zeglin, L.H., 2008. Stoichiometry of soil enzyme activity at global scale. Ecology Letters 11, 1252–1264.

Strickland, M.S., Osburn, E., Lauber, C., Fierer, N., Bradford, M.A., 2009. Litter quality is in the eye of the beholder: Initial decomposition rates as a function of inoculum characteristics. Functional Ecology 23, 627–636.

Stubberfield, L.C.F., Shaw, P.J.A., 1990. A comparison of tetrazolium reduction and FDA hydrolysis with other measures of microbial activity. Journal of Microbiological Methods 12, 151–162.

Tabatabai, M. a., Bremner, J.M., 1969. Use of *p*-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biology and Biochemistry 1, 301–307.

Tabatabai, M.A., 1982. Soil enzymes, in: Page, A.L., Miller, E.M., Keeney, D.R. (Eds.), Methods of Soil Analysis. Part 2. Chemical and Microbiological Properties. Soil Science Society of America, Inc. and American Society of Agronomy, Inc., Madison, pp. 903–947.

Tokuda, S.I., Hayatsu, M., 2002. Soil microbial biomass and fluorescein diacetate hydrolytic activity in Japanese acidic tea field soils. Soil Science and Plant Nutrition 48, 865–869.

Trevors, J.T., 1984. Dehydrogenase activity in soil: a comparison between the INT and TTC assay. Soil Biology and Biochemistry 16, 673–674.

Vance, E.D., Brookes, P.C., Jenkinson, D.S., 1987. An extraction method for measuring soil microbial biomass C. Soil Biology and Biochemistry 19, 703–707.

Wakelin, S., Lombi, E., Donner, E., MacDonald, L., Black, A., O'Callaghan, M., 2013. Application of MicroResp^{TM} for soil ecotoxicology. Environmental Pollution 179, 177–184.

Wang, Y.P., Shi, J.Y., Wang, H., Lin, Q., Chen, X.C., Chen, Y.X., 2007. The influence of soil heavy metals pollution on soil microbial biomass, enzyme activity, and community composition near a copper smelter. Ecotoxicology and Environmental Safety 67, 75–81.

Wilkinson, S.C., Anderson, J.M., Scardelis, S.P., Tisiafouli, M., Taylor, A., Wolters, V., 2002. PLFA profiles of microbial communities in decomposing conifer litters subject to moisture stress. Soil Biology and Biochemistry 34, 189–200.

Zeglin, L.H., Bottomley, P.J., Jumpponen, A., Rice, C.W., Arango, M., McGowan, A., Mfombep, P., Myrold, D.D., 2013. Altered precipitation regime affects the function and composition of soil microbial communities on multiple time scales. Ecology 94, 2334–2345.

Zhou, X., Wu, H., Koetz, E., Xu, Z., Chen, C., 2012. Soil labile carbon and nitrogen pools and microbial metabolic diversity under winter crops in an arid environment. Applied Soil Ecology 53, 49–55.

5. DIVERSIDAD Y FUNCIONALIDAD DE LOS HONGOS DEL SUELO

Resumen

La pérdida de biodiversidad producida por la contaminación puede afectar negativamente a los servicios ecosistémicos. La fitorremediación es una estrategia efectiva para gestionar grandes áreas contaminadas en las que puedan restaurarse los servicios ecosistémicos perdidos. El establecimiento de las comunidades de plantas en estos suelos podría mejorar la estructura de las comunidades de hongos y, en particular, apoyar el establecimiento de asociaciones simbióticas micorrícicas. La forestación de suelos degradados con diferentes especies de plantas puede promover el establecimiento de una diversidad y funcionalidad fúngicas determinadas.

En este estudio analizamos la diversidad de hongos y los gremios funcionales a través de técnicas de secuenciación masiva de ADN de suelos contaminados por elementos traza. Se seleccionaron cinco hábitats diferentes: tres bajo la copa de tres especies leñosas (acebuche, álamo blanco y pino piñonero), otro hábitat de suelos de pradera adyacentes y otro hábitat de suelos no remediados.

Las medidas de fitorremediación permitieron el establecimeinto de praderas de manera natural a lo largo del Corredor Verde del Guadiamar promoviendo la riqueza, diversidad, taxonomía y funcionalidad en comparación con los suelos no remediados. Además la forestación con árboles permitió que se estableciera una comunidad de hongos más similar a la de los bosques, con mayor taxonomía, principalmente de hongos ectomicorrícicos. Los efectos de estas especies fueron claros en el nitrógeno, carbono orgánico, calcio y relación C:N promoviendo una mayor heterogeneidad espacial con el potencial de aumentar la diversidad de hongos establecida.

La diversidad y la riqueza de las comunidades fúngicas del suelo aumentaron en los suelos tratados con medidas de fitorremediación. El álamo blanco fue la especie leñosa con las mayor diversidad y riqueza en comparación con el acebuche y el pino piñonero. En suelos remediados, las comunidades de hongos más diferentes se encontraron entre el pino piñonero y los suelos de pradera .

Se identificaron un total de 9428 OTUs que fueron referidos a grupos taxonómicos. Los hongos más abundantes fueron los saprótrofos, seguido de los patógenos y los ectomicorrícicos. Los hongos ectomicorrícicos fueron más dominantes en los suelos de las especies hospedadoras de estos hongos, mientras que los saprótrofos fueron abundantes en suelos de pradera y bajo acebuche. En los suelos no remediados los patógenos fueron los hongos más abundantes.

Abstract

Soil biodiversity loss due to pollution may affect ecosystem services negatively. Phytoremediation is an effective strategy to manage contaminated areas with the aim of restoring ecosystem services. Establishment of plant communities may improve fungal community structure and, in particular, establishment of mycorrhizal symbiotic associations. Afforestation of degraded lands may have different outcomes on fungal diversity and functionality depending on the selection of plant species to be used.

We analysed soil fungal diversity and functional guilds by high-throughput sequencing of environmental DNA in a trace element contaminated area, part of a large scale phytoremediation project running for 20 years. We selected five habitats for comparison purposes: three under the canopy of selected tree species (wild olive, white poplar and stone pine), one in the adjacent treeless areas (grassland) and one in the non-remediated areas (bare soil).

Soil fungal diversity and richness was enhanced by phytoremediation. White poplar soil had the highest diversity and richness compared to wild olive and stone pine. Fungal communities were especially different between stone pine, with soils rich in organic C and high C:N ratio, and grassland soils.

We identified 9,428 fungal OTUs that could be referred to taxonomic groups, the most abundant being saprotrophic, plant pathogenic and ectomycorrhizal functional guilds. Ectomycorrhizal fungi were enhanced by ectomycorrhizal host plants while saprotrophs and plant pathogens decreased in these soils. Saprotrophs were abundant in grassland and wild olive soils, while plant pathogens were abundant in non-remediated soils.

Phytoremediation allowed natural establishment of grassland habitats along the study area increasing fungal diversity, richness, taxonomy and functionality compared to non-remediated soils. Tree afforestation allowed establishment of a forest type community bringing a further recruitment of fungal taxa, mainly the ectomycorrhizal fungal guild. Afforestation with different tree species showed species-specific effects on soil N, organic C, Ca and C:N ratio which lead to increased spatial heterogeneity in areas with potential to recruit a wider diversity of fungi.

5.1 Introduction

Fungi are major components of the soil microbiota harbouring a wide diversity of life histories which deliver diverse ecological functions (Sun et al., 2017). Among their key functions, fungi are decomposers (saprotrophs), contributing to nutrient cycling; pathogens, regulating plant community composition; and mutualists (e.g. mycorrhizal), providing multiple services to plants (Nilsson et al., 2019; Tedersoo et al., 2014b; van der Heijden et al., 2008). However, there are evidence that soil fungal biodiversity declines as part of a global soil biodiversity loss due to human activities, causing negative effects on ecosystem services (Geisen et al., 2019; Veresoglou et al., 2015). Soil pollution is one of the main drivers of biodiversity loss and ecosystem change (Gardi et al., 2013; Rodríguez-Eugenio et al., 2018). Phytoremediation aims to recover contaminated land by using plants and their associated microorganisms to remove, detoxify and retain contaminants (Bolan et al., 2011) and has been proven as an effective strategy to manage trace element contaminated areas (Madejón et al., 2018; Wang et al., 2017). In general, restoration progress after ecosystem disturbances can be evaluated by measuring recovery of soil microbial diversity and activity (Banning et al., 2011; Bünemann et al., 2018). In the case of soils contaminated by trace elements, establishment of plant communities leads to changes in fungal community structure, and in particular, to establishment of symbiotic mycorrhizal associations (Op De Beeck et al., 2015; van der Heijden et al., 1998). Mycorrhizal symbiosis plays a critical role in water and nutrient uptake by host plants and enhances plant tolerance to trace elements in these harsh environments (Cabral et al., 2015; Smith and Read, 2008).

As plants are colonizing degraded areas, and organic matter and nutrients accumulate, the fungal community structure becomes more deterministic, and can be explained by environmental factors (Dini-Andreote et al., 2015). Development of fungal communities after afforestation of degraded lands may have different outcomes depending on the plant species used. Indeed, in forests, the establishment of different tree species increases fungal diversity as different tree species are associated with a characteristic fungal community (Unterseher et al., 2008). Among different plant lifeforms, trees may affect soil fungal communities in a wider extent than herbaceous species. Trees produce higher litter quantities and their root system are wider and deeper, creating heterogeneous environments for understory vegetation and fauna (Sun et al., 2016). Indeed, plant

lifeform and mycorrhizal type drive fungal communities in a wider extent than plant identity (Tedersoo et al., 2014b). Functional differences in fungal communities were observed between forested and treeless ecosystems, being ectomycorrhizae-dominated communities favored in forested soils (Sun et al., 2016; Tedersoo et al., 2014b, 2012). Tree composition has also been found to enhance specific fungal guilds, such as parasitic and mycorrhizal (Peay et al., 2013).

At the same time, abiotic factors also have direct and indirect (through affecting plant performance) effects on soil microbial communities. Among these factors, pH and nutrient content (N, P, Ca and C:N ratio) of soils have been repeatedly considered as principal regulators of soil fungal communities (Narendrula-Kotha and Nkongolo, 2017; Sun et al., 2016; Tedersoo et al., 2014b). However, in disturbed areas, soil fungal communities have shown a higher correlation with plant community composition than with soil abiotic properties (Mueller et al., 2014).

Studying the relative contribution of plant identity and soil properties in mining soils represent an opportunity to understand how microbial communities develop in soils dramatically degraded. Indeed, mining related activities have produced land disturbances around the world, generating severe contaminated areas, that have motivated some large scale phytoremediation projects (Pulford and Watson, 2003; Wang et al., 2017). However, studies addressing interactions between plant species identity and initial soil factors are scarce (Berg and Smalla, 2009; de Vries et al., 2012; van der Linde et al., 2018) and hence the ecological basis for design of successful phytoremediation strategies are still incomplete. More knowledge is needed to understand the importance of the plant species used in phytoremediation activities and the necessary conditions for plant establishment.

In this study, we analyzed soil fungal diversity and functional guilds in a trace element contaminated area, afforested 20 years prior to sampling, as part of a large-scale phytoremediation project. We selected patches with different vegetation covers (soil covered by trees species, herbaceous plants or bare soils), corresponding to different degrees of intervention during implementation of the project, in order to understand how vegetation affects composition and diversity of soil fungal communities. Bare soils correspond to areas where soil contaminants were not removed or effectively remedied with organic matter or calcium-rich amendments; grassland areas were the result of actively cleaned and amended soils, but were not afforested and were colonized by natural

herbaceous species; soils underneath trees were actively cleaned, amended and afforested. We hypothesized that 20 years after restoration started, (i) soil fungal diversity would increase with the degree of intervention, (ii) composition of soil fungal communities would be affected by both vegetation cover (in particular of tree identity) and soil properties and (iii) fungal functional diversity, with special focus on mycorrhizal communities, would be mostly influenced by tree identity. Elucidating these points would help to better evaluate the success of managing restoration of contaminated soils.

5.2. Materials and methods

5.2.1. Study area and tree species

We repeated the sampling design in two sites; the North and the South site (Fig. S5.1). See the general description of the study site in chapter 3.

The afforestation program implemented in 1999-2000 included planting saplings of more than 40 woody species with a density of 700-900 plants ha^{-1}, in a mixed design to simulate natural forest conditions. Planted saplings had to resist summer drought, high irradiance, altered soil structure, residual contamination and competition from herbaceous plants, which resulted in a high mortality of some species the first years after planting (Domínguez et al., 2010). Eighteen years after afforestation, we selected three tree species well established in both sites and with contrasted life-history strategies: wild olive (*Olea europaea*), white poplar (*Populus alba*) and stone pine (*Pinus pinea*).

There were size differences among tree species. Wild olive tree specimens were smallest in height and crown projection, and their growth was more reduced at the North site (Table S5.1). White poplar was the biggest tree species at the North site and stone pine at the South site.

5.2.2. Sample design, collection and processing

At both sites, North and South, we sampled soil fungal communities in five habitat types, i.e. plant covers. Three habitats were under the canopy of the selected tree species (wild olive, white poplar and stone pine). The fourth habitat was the adjacent treeless areas where natural grassland was established. For comparative purposes, we also sampled a fifth habitat. At the North site we sampled soils devoid of vegetation, bare soils, where soil amendment addition was not effective (García-Carmona et al., 2019b). At the South site, we sampled bare soil patches in a non-remediated area, covered by mining sludge, which was left for research purposes (Burgos et al., 2013).

In March 2018, topsoils were collected (0-5 cm depth) with a gouger auger; five soil cores were taken under the tree canopy and mixed to make one composite sample per tree. A similar area was sampled in the grassland and bare soils habitats. We sampled five replicates for each habitat or plant cover type (wild olive, white poplar, stone pine, grassland and bare soil (without plant cover)), and repeated the sampling at the North and South sites, making a total of 50 sampled soils (Fig. S5.1). Replicates were separated by at least 30 m. Soil samples were taken to the laboratory and kept stored overnight at 4 ºC until next day when soil was processed.

5.2.3. Soil analyses

A fraction of the soil samples was air-dried and sieved to < 2 mm for chemical analyses. Soil pH was measured in a 1:2.5 soil- KCl 1 M suspension after 30 min of shaking. Total C and N was determined by a Flash HT Plus elemental analyser. Carbonate content was measured by the manometric method (Demolon and Leroux, 1954). The difference between total C and carbonates provided organic C content. Total N was assumed to be organic in order to calculate the C:N ratio, with the organic fractions. Available P was estimated by the Olsen method. X-Ray fluorescence (XRF) spectroscopy (XRF Niton™) was used to measure total soil elements (Ca, K, Zr, Sr, Rb, Pb, As, Zn, Cu, Fe, Mn, Cr, V, Ti and S). Particle size distribution was determined by the hydrometer method (Gee and Bauder, 1979).

A second fraction of the soil samples was kept fresh at 4 ºC for one week and sieved to < 2 mm for microbial analyses; we expect little effects of storage conditions on fungal community structure (Rubin et al., 2013). Dehydrogenase activity was determined following the method proposed by Trevors (1984). Soils were incubated with iodonitrotetrazolium (INT) at a concentration of 0.4% (w/v), in a 1 M TRIS-HCl buffer (for optimal pH 7.5), in the dark at 20 ºC for 20 hours. After incubation, 10 ml of methanol were added and shaken for 1 minute and samples were filtered. The production of p-iodo-nitrotetrazidin formazan (INTF) produced during the incubation was measured by spectrophotometer, determining absorbance at 490 nm in the extracts. For basal respiration analysis we used MicroResp^{TM} method (Campbell et al., 2003), adding water for basal respiration measurement. Before measurement, soils were incubated for a week at 20 ºC with a 30-60% WHC to settle microbial communities after sampling and processing disturbance.

A third fraction of the soil samples for molecular analysis was frozen at -80 ºC.

5.2.4. Molecular analyses

Total DNA was extracted from the 50 soils samples with the PowerSoil® DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA), in accordance with the manufacturer's instructions. From each soil sample two DNA extractions were carried out resulting in a total of 100 sequenced samples. Library preparation and Illumina sequencing were performed by the IPBLN Genomics Core Facility (CSIC, Granada, Spain). Amplicon libraries targeting the ITS1 region were generated by a two-step PCR strategy. Gene-specific amplification was performed in triplicate with 10 ng of soil-extracted DNA in a final volume of 10 µl. Primers ITS1F-FW (5´-CTTGGTCATTTAGAGGAAGTAA-3´, Gardes and Bruns, 1993) and ITS2-Rev (5´-GCTGCGTTCTTCATCGATGC-3', White et al., 1990) were designed with Nextera overhang adapters and used at 0.3 µM final concentration. These fungal primer pairs are recognized as the primary barcode for fungi (Schoch et al., 2012), however they are biased to Ascomycota and Basidiomycota (Bellemain et al., 2010). Reaction was performed with 1X KAPA HiFi HotStart ReadyMix DNA polymerase (Roche Diagnostics, West Sussex, United Kingdom). Cycling conditions were 95°C for 3 min, 32 PCR cycles; 95°C for 30

s, 52°C for 30 s, 72°C for 30 s and then 72°C for 5 min. 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 PCR cycles; 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and then 72°C for 5 min. Concentration was measured on the Qubit® fluorometer (Thermo). Amplicons were diluted and 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 300x2 nt paired-end sequencing was conducted on a MiSeq sequencer.

5.2.5. Bioinformatic analyses

We analyzed the MiSeq sequences with amplicon sequence variant analysis pipeline, known as DADA2 v. 1.8. (Callahan et al., 2016). Briefly, forward and reverse sequences were trimmed to 260 bp, primers removed and a quality score set up to two. Sequences were dereplicated and the rate error model inferred and used to implement the sample inference algorithm. Forward and reverse reads were merged and chimeric sequences removed, accounting for a total of 20,320 amplicon sequence variants (ASVs). The taxonomic assignation was determined for each ASV against the UNITE database v. 7.2. (Kõljalg et al., 2013; UNITE Community, 2017). Non-fungal or unknown taxa (6%) were removed, and 19,098 ASVs were kept. Afterwards, post-clustering curation tool, known as *LULU* (Frøslev et al., 2017), was used to remove erroneous ASVs (a total of 4,125). Finally, ASVs were clustered at 97% sequence similarity using VSEARCH v. 2.8.1. (Rognes et al., 2016) to finally define 9,428 Operational Taxonomic Units (OTUs). Functional guilds were determined using FunGUILD (Nguyen et al., 2016) and only 2,309 OTUs (24.5% of total) could be assigned. After that first assignment we only kept those OTUs with probable or highly probable confidence ranking, and having only one guild assigned; as result we retained 1,283 OTUs (13.6% of total) for the functional analysis. Raw sequences were deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) under BioProject identification number

PRJNA607425 and BioSamples identification numbers SAMN14132727 - SAMN14132924. Bioinformatic analyses were carried out in R software v. 3.6.1 (R development Core Team) using *dada2* (Callahan et al., 2016), *LULU* (Frøslev et al., 2017) and *phyloseq* (McMurdie and Holmes, 2013).

5.2.6. Statistical analyses

An OTU abundance data matrix was constructed based on the number of reads assigned to the 9,428 fungal OTUs identified. Rarefaction curves were visualized to ensure a correct coverage of the total diversity of OTUs in sampled soils. Since every sample reached plateau, there was no need to rarefy to a minimum number of reads per sample (McMurdie and Holmes, 2014).

We calculated Shannon´s diversity (H), Pielou´s evenness (J), OTUs richness (S_{OTU}) and family richness (S_f) for each sample. Beta diversity was calculated using Whittaker´s betadiversity (W). Gamma (landscape) diversity of each site was the cumulative diversity of the five sampled habitats and was illustrated by Venn diagrams (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Differences in fungal diversity indexes, as well as in soil abiotic and biotic variables, between plant cover type (habitat), sampling site and their interactions were tested by two-way analysis of variance (ANOVA); when they were significant, the multiple comparison between means was tested by Tukey´s honest significant different (HSD) *post hoc*. Statistical significance threshold was fixed at $p \leq 0.05$. We tested normality with Shapiro–Wilk test, and homoscedasticity with Levene´s test. When assumptions were not met, data was Box-Cox transformed or a constant variance function (*varIdent* function) was performed. A non-parametric Scheirer–Ray–Hare test and a Dunn´s test corrected by Bonferroni *post hoc* were performed when data did not meet the assumptions. In order to explore the relationships between fungi and soil variables, Pearson's correlation tests (adjusted with Benjamini-Hochberg correction) were performed.

We tested plant cover and site factors effects on the soil fungal community composition by means of a permutation multivariate analysis of variance (PERMANOVA) implemented with adonis function (1,000 permutations). Differences in multivariate dispersion between factor levels, that could affect the results of PERMANOVA, were

addressed by testing the homogeneity of variances (betadisper function). The revealed trends by PERMANOVA (effects of plant cover and site) were represented in a non-metric multidimensional scaling (NMDS) ordination biplot. In the NMDS, we fitted significant ($p < 0.05$) soil variables with the envfit function to know the variables covarying with the fungal community structure. In addition, partial NMDS analyses were carried out separately with North and South samples to better illustrate the plant cover effects. All these analyses used Bray-Curtis distance as the measure of dissimilarity.

We conducted an indicator species analysis (ISA) on the OTUs absolute abundance dataset (*multipatt* function) obtaining specific OTUs, through a permutation test with 999 permutations, tied to specific factor levels (Dufrêne and Legendre, 1997). The indicator values were classified according to plant cover and site factors.

Statistical analyses were carried out in R software v. 3.6.1 (R development Core Team) using *indicspecies* (De Cáceres and Legendre, 2009), *ggplot2* (Wickham, 2016), *lme4* (Bates et al., 2015), *nlme* (Pinheiro et al., 2016), *phyloseq* (McMurdie and Holmes, 2013), and *vegan* (Oksanen et al., 2016) packages.

5.3 Results

5.3.1 Soil fungal diversity

A total of 13 fungal phyla were found. Ascomycota, Basidiomycota, Mortierellomycota and Chytridiomycota were the most abundant (Fig. 5.1). The minimum number of phyla (only four) was found in the non-remediated bare soils of the South site, in contrast with the average of 11.5 phyla in remediated soils covered by vegetation from the same site.

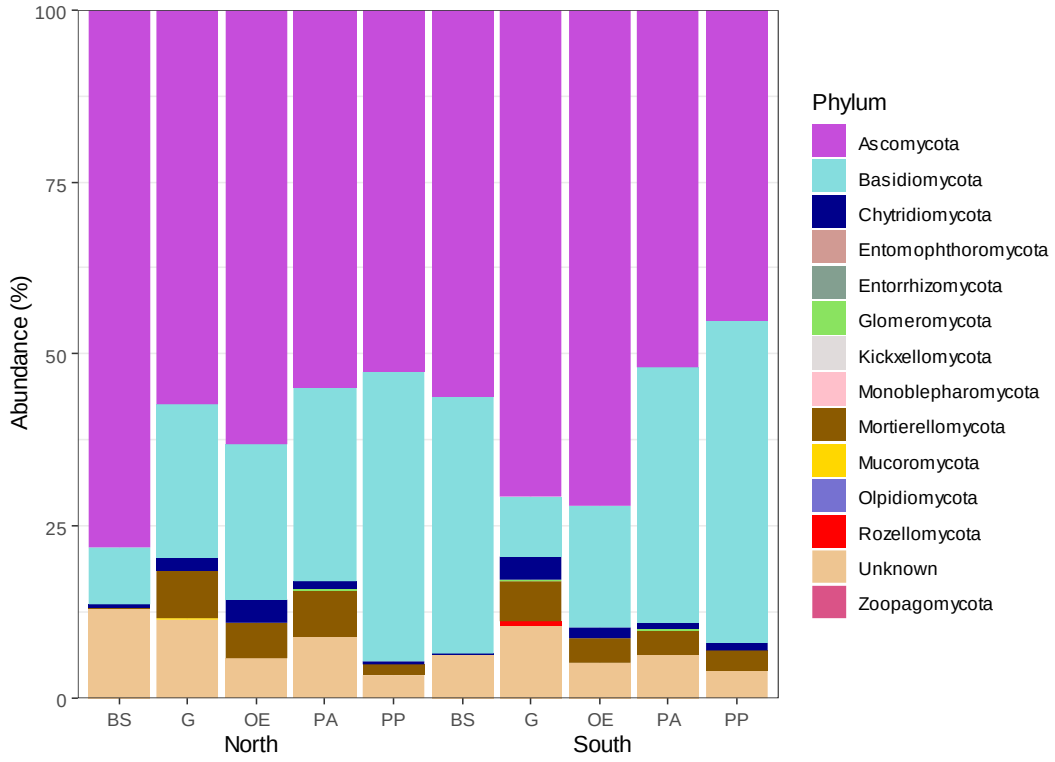


Figure 5.1 Relative abundance of fungal phyla in the 10 habitat types according to plant cover (BS = Bare soil, G = Grassland, OE = *Olea europaea*, PA = *Populus alba*, PP = *Pinus pinea*) and site (North, South) factors.

In bare soils, the number of different families (S_f, family richness) was significantly lower than in the remediated soils (Table 5.1). The analysis of the top abundant families (n = 20) showed a large variation between sites and vegetation types (Fig. S5.2); these 20 families represented more than 75% of the relative abundance in bare soils (Fig. S5.2). Grassland soils presented a higher number of families compared to bare soils and they were highly even in terms of abundance. Families recorded in wild olive tree soils were similar to grassland soils, except for a characteristic dominance of *Trichomeriaceae* and *Pyronemataceae*. Soils covered by poplar and pine were richer in fungal families, with a high dominance of *Thelephoraceae, Inocybaceae, Tricholomataceae* and *Atheliaceae.*

Plant cover had a significant influence on the diversity of soil fungal communities (Table 5.1). Species richness (S_{OTU}) was the lowest in bare soils and the highest in white poplar soils, being intermediate in the other plant covers. Shannon (H) index weas also lowest in bare soils but highest in soils covered by grassland and wild olive. Site factor did not have a significant effect for Pielou´s evenness, though a significant interaction was seen between plant cover and site. Fungal communities were most even in grassland soils at the North site and underneath wild olive and bare soils at the South site. Among habitats,

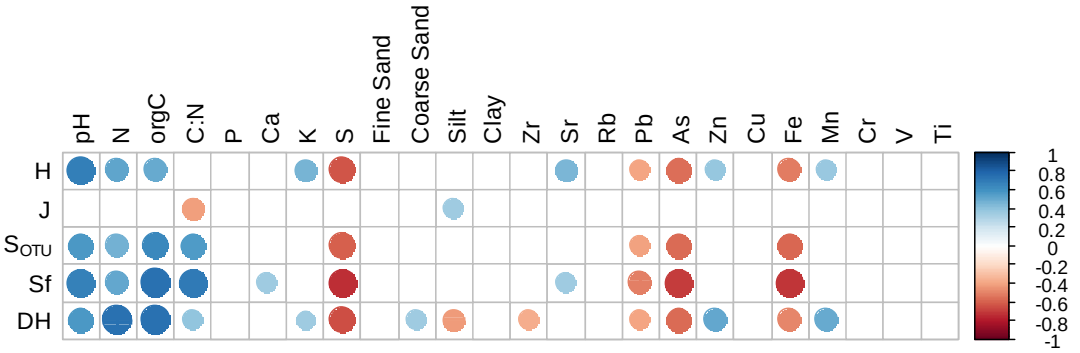
betadiversity (W) was reduced in grassland and white poplar soils at the South site (Table 5.1).

There was a significant relationship between fungal diversity indexes and some soil properties (Fig. 5.2). When including non-remediated soils (n = 50), pH, N, organic C and C:N ratio showed a positive correlation with most of the indexes, while it was negative with variables related to soil contamination, such as S and some trace elements (As, Fe and Pb) (Fig. 5.2, A). When only soils covered by vegetation were considered (n = 40), C:N ratio had the most negative effect on Shannon and Simpson´s indexes, while organic C had the most positive effect on richness (Fig. 5.2, B). Pielou´s correlated negatively only with C:N ratio in correlation analyses of both data sets.

Table 5.1 Mean ± SE and two-way analysis of variance (ANOVA) statistic F with effects of plant cover (Bare soil, Grassland, *Olea europaea*, *Populus alba* and *Pinus pinea*) (*df* = 4), site (North and South) (*df* = 1) and their interaction (*df* = 4) on fungal biodiversity indexes (n = 50). Tukey's honest significant difference (HSD) *post hoc*. Uppercase letters indicate differences across plant cover types (sites were summed up). When a significant interaction was detected, differences across plant cover types and sites were indicated by lowercase letters. Asterisks indicate the significance level (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$). δ: Non-parametric Schneirer-Ray-Hare test and H statistic; α: *VarIdent* function used to meet the homoscedasticity assumption. H: Shannon index; J: Pielou's evenness; S_{OTU}: OTUs richness; S_f: Family`s richness; W: Whittaker's betadiversity.

Index	**Site**	**Plant cover**					**ANOVA**			
		Bare soil	Grassland	*Olea europaea*	*Populus alba*	*Pinus pinea*	Plant cover	Site	Interaction	
H	North	2.97±0.21**C**	4.75±0.20**A**	4.53±0.10**A**	4.70±0.12**A**	3.87±0.30**B**	**30.80*****	0.49	1.03	α
	South	2.95±0.5	4.83±0.12	4.69±0.13	4.16±0.25	3.78±0.25				
J	North	0.533±0.032**bc**	0.715±0.015**a**	0.693±0.013**ab**	0.675±0.016**ab**	0.564±0.047**abc**	**13.32*****	0.48	**10.40*****	α
	South	0.765±0.028**a**	0.692±0.009**ab**	0.762±0.020**a**	0.591±0.039**abc**	0.545±0.024**c**				
S_{OTU}	North	303±72**C**	835±198**AB**	706±70**B**	1076±113**A**	995±113**AB**	**15.88*****	0.03	1.04	
	South	51.2±9.0	1095±101	599±234	1168±99	1069±193				
S_f	North	62.6±9.7**c**	95.6±8.0**ab**	101.8±5.5**ab**	124.0±6.6**ab**	123.8±6.6**ab**	**32.02*****	0.05	**3.74***	
	South	19.8±1.9**d**	111.0±5.5**ab**	95.0±18.1**bc**	141.0±6.8**a**	134.6±12.4**ab**				
W	North	0.429±0.041**ab**	0.430±0.029**a**	0.377±0.010**a**	0.388±0.017**a**	0.405±0.015**a**	**4.97****	<0.01	**2.70***	
	South	0.424±0.026**a**	0.330±0.012**c**	0.410±0.030**a**	0.334±0.013**bc**	0.364±0.011**ab**				

A



B

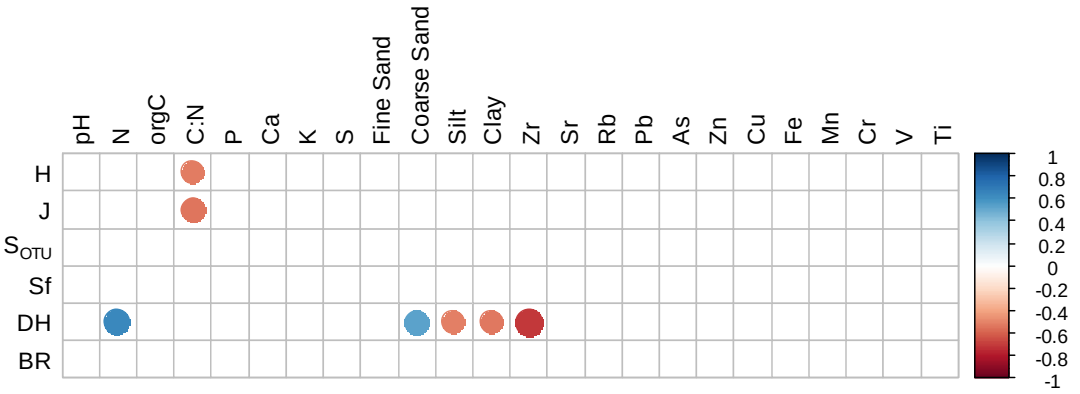


Figure 5.2 Pearson´s correlation coefficient between diversity indexes, dehydrogenase (DH) and basal respiration (BR) and soil abiotic variables A) including bare soil (n = 50) and B) excluding bare soil (n = 40). Circles indicate significant differences ($p < 0.05$); blue and red colours indicate positive and negative correlation, respectively.

The differences in fungal communities among habitats is reflected by the indicator analysis (Table S5.2) and the Venn diagram (Fig. 5.3). From a total of 9,428 OTUs, 1,351 (14%) were “indicators” (that is, specific) of a type of soil in terms of site and plant cover factors (Table S5.2, A). A list of fungal OTUs indicators of the extreme conditions in the non-remediated bare soils, covered by mine sludge can be found in supplementary material (Table S5.2, B).

The Venn diagrams were different between North and South sites (Fig. 5.3). Bare soils at the South site had only 75 unique fungal OTUs and shared 2% of these OTUs with plant covered soils. Among the remediated and vegetated soils, wild olive had the lowest

proportion of unique OTUs (6% at both sites) while white poplar soils at the North (17%) and grassland soils at the South (17%) had most unique OTUs.

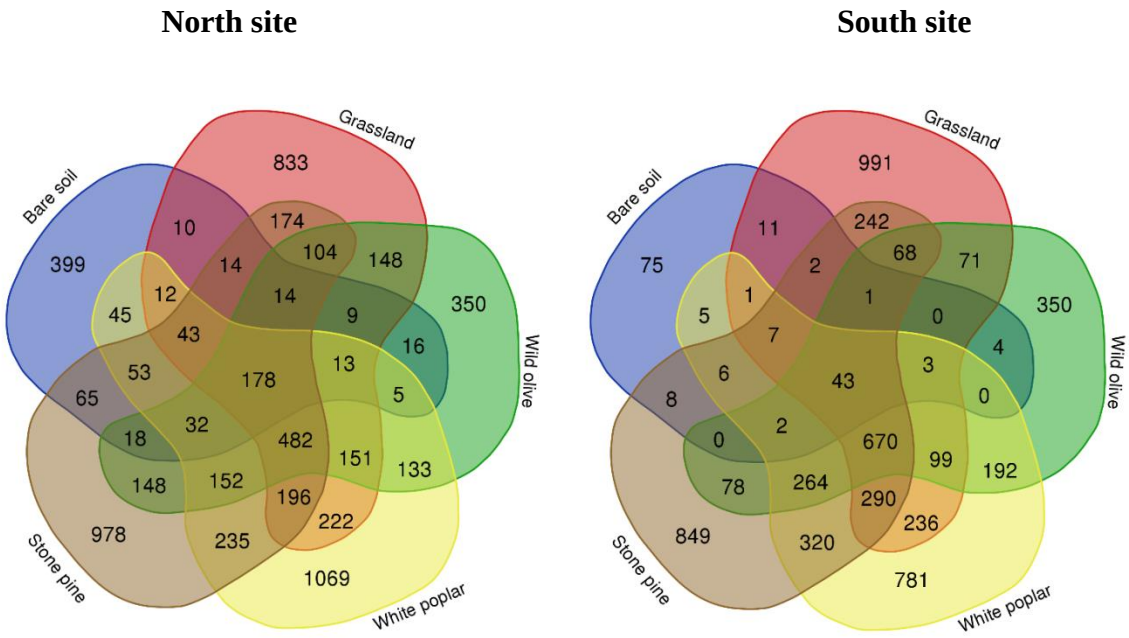


Figure 5.3 Venn diagrams of operational taxonomic units (OTUs) distribution among the samples according to plant cover factor. Numbers indicate the number of unique or shared OTUs. Each diagram is a different site, North and South.

There were clear differences in the total number of OTUs among plant covers, but they were very similar between sites (Table S5.3). Wild olive soils supported the lowest number of fungal OTUs and the white poplar soils the highest. The landscape (gamma) diversity of the soil mycobiota was over 5,000 OTUs at both sites.

5.3.2. Soil fungal community composition and soil properties

In the 50 soil samples we found a total of 9,428 fungal OTUs, and the global species accumulation curve was close to saturation (Fig. S5.3, A). The number of sequences was sufficient to represent the fungal diversity in this study (Fig. S5.3, B).

Plant cover and site factors and their interaction, as shown by PERMANOVA and NMDS ordination (Table 5.2 and Fig. 5.4) significantly affected the composition of soil fungal

communities. The first axis of the NMDS ordination showed a clear separation between soil fungal communities from the North site, characterized by high availability of trace elements, lower pH and sandy texture, and those from the South site, characterized by higher pH, Ca, K and P contents and dominant clay and silt particles. The second axis of the NMDS ordination separated fungal communities according to plant cover. The most pronounced difference in fungal communities was seen between stone pine, with soils rich in organic C and high C:N ratio, and grassland fungal communities (Fig. 5.4). Stone pine fungal communities were discriminated by partial NMDS at both North and South sites (Fig. S5.4), although the correlated abiotic variables were different. A PERMANOVA analysis for each site separately showed that the plant cover effect on fungal communities was higher at the South site (R^2 = 0.38) compared to the North site (R^2 = 0.27) (Table 5.2).

Table 5.2 Permutational multivariate analysis of variance (PERMANOVA) of the effect of plant cover and site on the distribution of fungal communities (n = 40) in soil. In bold, statistically significant relationships ($p < 0.05$).

	Df	**SS**	**MS**	**F.Model**	**R^2**	**Pr (>F)**
Plant cover	3	2.55	0.85	3.44	0.191	**< 0.001**
Site	1	1.56	1.56	6.33	0.117	**< 0.001**
Interaction	3	1.35	0.45	1.82	0.101	**< 0.001**
North site						
Plant cover	3	1.55	0.52	2.00	0.272	**< 0.001**
South site						
Plant cover	3	2.35	0.78	3.33	0.385	**< 0.001**

Df, degrees of freedom; SS, sum of squares; MS, Mean squares; Pr value by permutation.

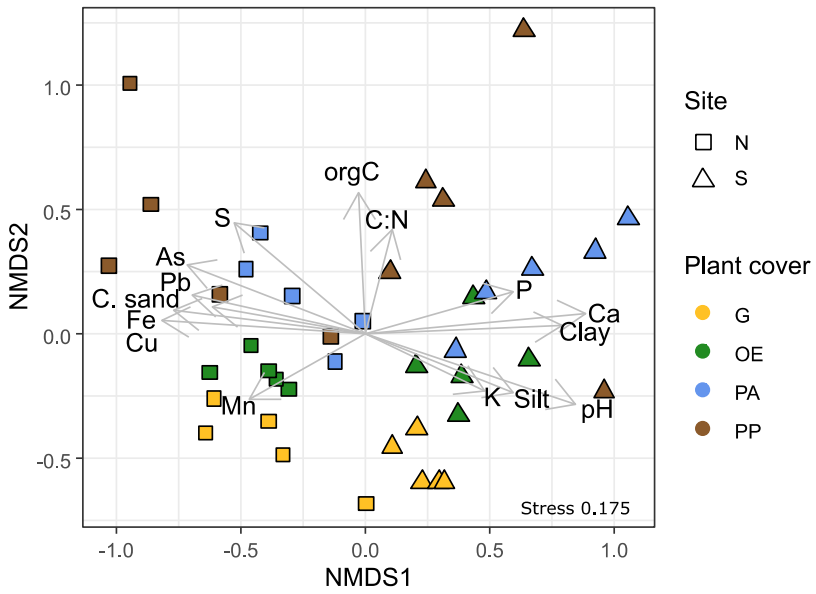
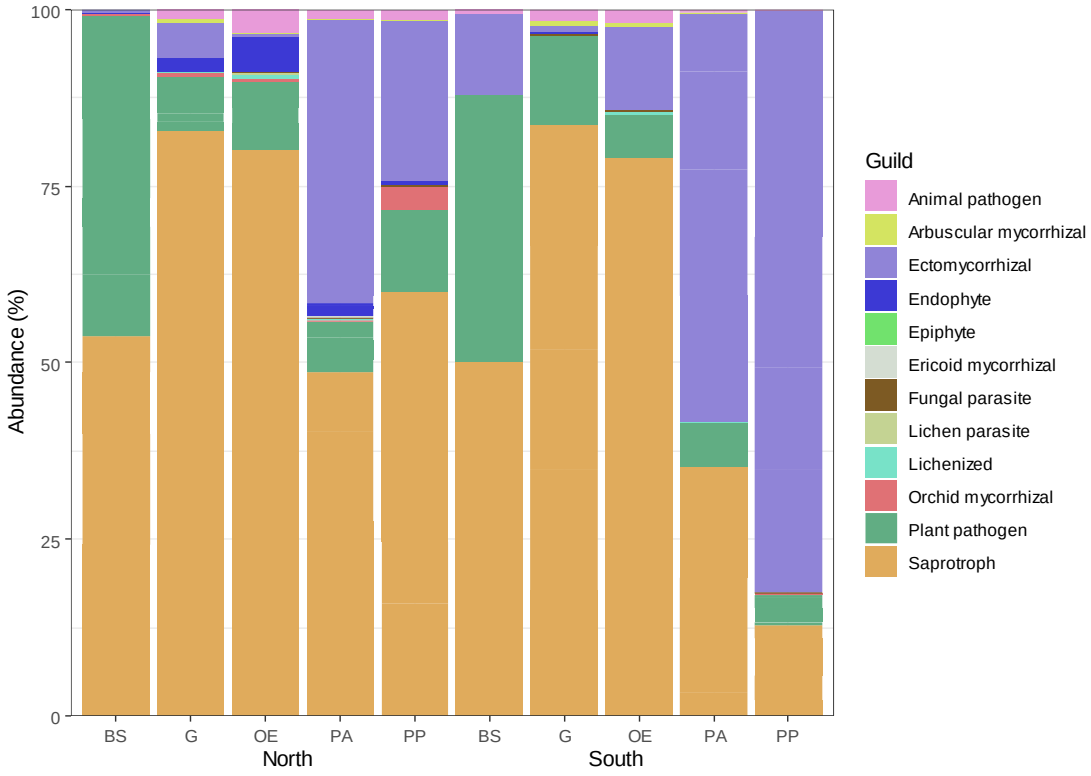


Figure 5.4 Non-metric multidimensional scaling (NMDS) ordination biplot of fungal communities. Symbols according to site (N = North (square) and S = South (triangle)) and plant cover (G = Grassland, OE = *Olea europaea*, PA = *Populus alba*, PP = *Pinus pinea*). Significant ($p < 0.05$) soil properties are presented as arrows.

5.3.3. Fungal functional diversity

In terms of relative abundance, saprotrophs was the most abundant functional group, especially in grassland and wild olive soils (Fig. 5.5, A). The second one was ECM fungi which were mainly found in white poplar and stone pine soils, with over 50% abundance in the South site (Fig. 5.5, B). The third most abundant guild, plant pathogen fungi, was highly abundant in bare soils, and their abundance was significantly reduced in the rest of plant covers (ANOVA results were $F = 17.26$; $p < 0.001$). In contrast to these three major guilds, arbuscular mycorrhizal fungi had low abundance in all studied soils. In terms of OTUs richness, saprotroph guild was the richest (68 %) followed by plant pathogen and ECM guilds (Table S5.4). However, in terms of total reads, ECM guild accounted for over 50% of total reads, followed by saprotrophs and plant pathogens (Table S5.4). We studied the relationships between the abundance of these three guilds and we found significant and negative correlations were found of ECM with saprotrophs (r = -0.85; $p < 0.001$) and plant pathogens (r = -0.40; $p = 0.004$); while no significant correlation was seen between saprotrophs and plant pathogens (r = -0.13; $p = 0.365$).

A



B

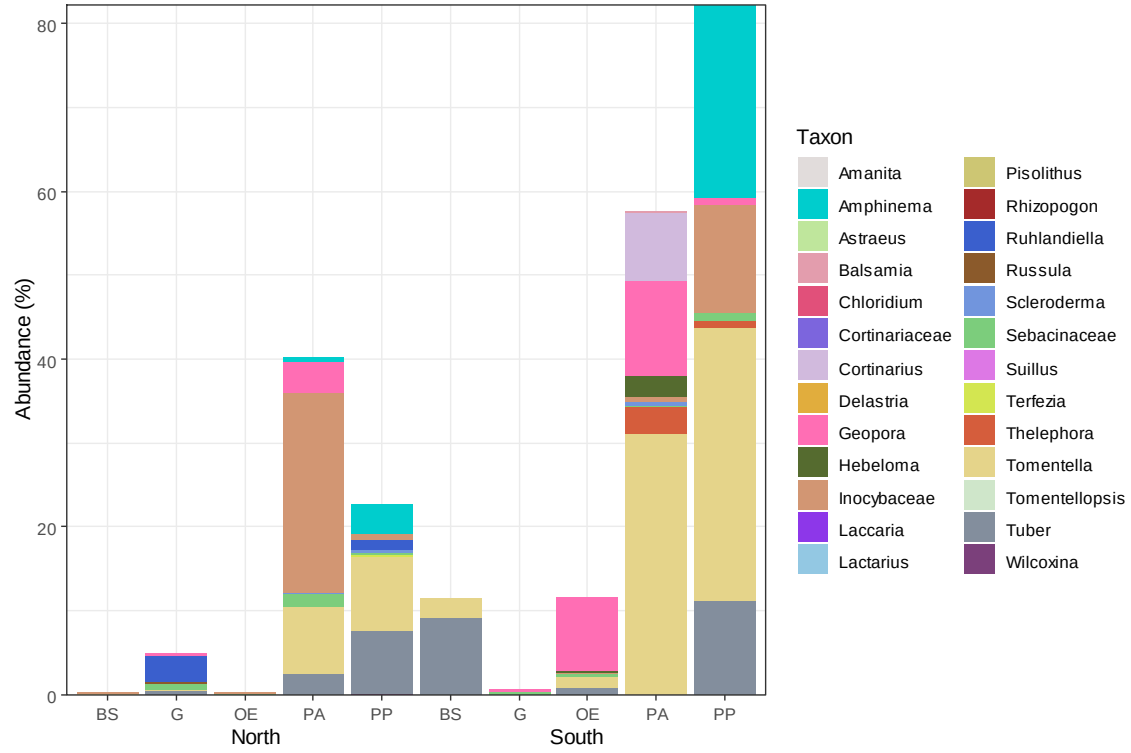


Figure 5.5 Relative abundance of A) fungal guilds and B) ECM fungal taxa in the 10 habitat types according to plant cover (BS = Bare soil, G = Grassland, OE = *Olea europaea*, PA = *Populus alba*, PP = *Pinus pinea*) and site (North, South) factors.

The abundance of functional groups was related to soil variables (Fig. S5.5). Thus, the abundance of saprotrophic and ECM fungi showed a positive correlation with C:N ratio; the abundance of ECM fungi was correlated also with soil Ca content. Finally, the abundance of arbuscular mycorrhizal fungi showed a positive correlation with pH and content of Ca and clay, while correlated negatively with Cu concentration.

Focusing on the composition within the guild of ectomycorrhizal fungi, we applied a separate PERMANOVA analysis. Ectomycorrhizal fungal composition was assessed by PERMANOVA showing significantly different communities in terms of plant cover (F = 2.20; p = 0.001; R^2 = 0.145), site (F = 2.15; p = 0.002; R^2 = 0.047) and their interaction (F = 1.57; p = 0.001; R^2 = 0.104). These results were similar to those obtained for all fungal communities (Table 5.2). Ectomycorrhizal communities were more clustered under white poplar and stone pine communities than under grassland and wild olive ones (Fig. S5.6). Samples of bare soils, which did not present an important ECM community, were excluded from this ECM analysis.

5.3.4. Soil characteristics of fungal habitats

Soil properties were significantly different in terms of plant cover and site, however no interaction between these two factors was found (Table S5.5). Soil pH was significantly different between sites. In the North site soils were slightly acidic in grassland areas, underneath wild olive and white polar, and strongly acidic under stone pine. In the South site, all plant-covered soils were close to neutral. In contrast, bare soils in both sites were strongly acidic. Calcium content was significantly higher in the South site and under white poplar compared to grassland and stone pine. Nitrogen content was significantly higher under white poplar than in grassland soils. Organic C and C:N ratio were found to be higher under white poplar and stone pine, when compared to grassland. In the South site, P, Zr and Sr total concentrations were higher, while Pb, As, Cu, Fe, Mn, Cr, V, Ti and S were significantly higher in the North site. Dehydrogenase activity in the North site was significantly higher than in the South site. Soil K, Rb, Zn and basal respiration did not differ between the studied soils. Soil texture was significantly different between sites. The South site presented higher proportion of fine fractions (fine sand, silt and clay), while the North site registered a high proportion of coarse sand.

5.4 Discussion

The recovery of soil microbial diversity is essential for restoration of degraded lands (Banning et al., 2011; Bünemann et al., 2018). In phytoremediated areas, the effects of establishing plant communities on soil microbiota is especially important in fungal communities due to their symbiotic mycorrhizal associations (Op De Beeck et al., 2015; van der Heijden et al., 1998). Therefore, the plant species selected in phytoremediation, as well as their lifeform, may be of critical importance in the restoration of soil biodiversity after a large perturbation.

5.4.1 Soil remediation and revegetation increases fungal diversity

In this study, fungal diversity and richness were enhanced in phytoremediated soils, probably due to the plant effect on increasing soil pH, N, organic C and Ca.

In non-remediated soils, the adverse abiotic conditions and the scarcity of nutrients did not boost fungal diversity and richness. Previous studies in the area found that acidic pH and high trace element availability were detrimental for AM (Montiel-Rozas et al., 2016) and ECM fungal diversity. White poplar and stone pine promoted richer fungal communities than wild olive soils probably due to the symbiosis with ECM fungi and higher organic C content (Tedersoo et al., 2014a). In agreement with Saitta et al. (2018) who found that pine trees reduced fungal diversity; in our study, stone pine reduced diversity and evenness, indicating dominance by a few species. This was likely related to the negative effect of a due to the negative effect of a high C:N ratio on Shannon diversity and evenness, also reported by other authors (Ni et al., 2018). This effect of pine trees might be related to the acidifying effect on soil surface underneath pine, likely caused by the leaching of organic acids during the decomposition of pine litter (Madejón et al., 2018; Richardson, 2000). Acidification of soils underneath pine trees occurred at the acidic site (North site), with a lower soil carbonate content; this acidification effect may limit colonization/growth of some fungal groups (Tedersoo et al., 2014b; Urbanová et al., 2015).

Different habitats established a unique distribution of OTUs related to differences in soil properties across plant cover types. This habitat effect has also been reported in several

works (Meiser et al., 2014; Rincón et al., 2015). Despite significant differences in OTU composition, the OTUs richness was consistent between the two sites. Ectomycorrhizal fungal hosts (white poplar and stone pine) registered a higher number of OTUs and presented less variable fungal communities due to their symbiosis with ECM fungi. In contrast, wild olive had the lowest number of OTUs and a high overlapping of OTUs with other vegetated habitats. This habitat also showed the highest betadiversity, which may indicate a fungal community more dependent on the environment and affected by the soil contamination. Despite the contrasted soil conditions, the gamma diversities of fungal communities in vegetated habitats were similar. These results contribute to the knowledge of soil microbial diversity at multiple scales (Walters and Martiny, 2020).

5.4.2. Tree species identity and abiotic soil conditions influence fungal community composition

We found that the effect of plant cover was the main driver of the composition and structure of soil fungal communities. In accordance, soil fungal communities have been seen strongly determined by dominant vegetation (Buée et al., 2009; Urbanová et al., 2015). As exposed above, and as found in many other works (Berg and Smalla, 2009; Chaparro et al., 2014; Leff et al., 2018), this significant influence may be related to specific changes on the abiotic/biotic environment below each tree, i.e. the “footprint”. In our study, the observed changes in soil organic C and C:N ratio across plant covers were the main drivers of the soil fungal communities.

Differences in soil pH and trace element concentration had an important effect on soil fungal communities, in accordance with previous evidences (Rincón et al., 2015). Different soil abiotic properties presented different effects on fungi: soil nutrients and C contents were key drivers of fungal diversity, richness and evenness, while soil pH, texture and trace element contamination had an effect on taxonomic and functional composition of the fungal communities.

5.4.3. Some fungal groups are more tolerant to acidic and metal-rich soils

Basidiomycota is often dominant in forest soils (Buée et al., 2009; O'Brien et al., 2005; Sun et al., 2016; Tedersoo et al., 2014b; Zappelini et al., 2015). However, in our study site, Ascomycota was the most abundant phylum. This pattern has been observed previously in another trace element contaminated site (Narendrula-Kotha and Nkongolo, 2017) and in soils under poplar tree species both from polluted (Foulon et al., 2016) and unpolluted areas (Shakya et al., 2013). The process of litter degradation tends to replace Ascomycota fungi, which selectively degrade cellulose over lignin, by Basidiomycota phylum, which synthetize enzymes able to degrade complex polymers (Djemiel et al., 2017; Sun et al., 2016; Voříšková and Baldrian, 2013). In the pine soils of this study, Ascomycota were somehow replaced by Basidiomycota, which may be explained by the low quality of pine litter (Dickie et al., 2014; Finzi et al., 1998; Wurzburger and Hendrick, 2007). Moreover, this replacement is usually followed by a functional shift from saprobic to mycorrhizal taxa (Peršoh, 2015) and we certainly observed an ECM fungal dominance (which belong predominantly to the Basidiomycota phylum (Tedersoo et al., 2010)) in the soils underneath pines.

The high percentage of indicator species may indicate a strong habitat filtering (Dufrêne and Legendre, 1997). For example, in the most acidic and non-remediated habitat, specific taxa were indicative of these extreme conditions. *Malasseziaceae* and *Terathosphaeriaceae* families have been recognized as highly tolerant to some of the most extreme environmental conditions, such as extremely acidic pH and high temperatures (Amend, 2014; Hujslová et al., 2013). Moreover, the indicator species *Coniochaeta fodinicola* and *Fodinomyces uranophilus* have been found by Vázquez-Campos et al. (2014) in an Uranium mine ecosystem in very acidic soils with high concentrations of Al and Fe. These findings support the existence of extremophile fungi in the adverse conditions that prevail in the non-remediated and bare soils of the study area. We expect that these communities of extremophile fungi would be replaced by other fungal species with greater competitive capacities, when soil conditions improve after active remediation and revegetation (Hujslová and Gryndler, 2019).

In strongly acidic and highly contaminated soils, *Tuber sphaerospermum* and *Tomentella sp*. were the only ECM fungi able to establish. *Tuber sphaerospermum* is known for their suitability to settle in acidic soils (Alvarado 2012), despite the preference of high pH and

Ca content for this taxon (Iotti et al., 2010). *Tomentella* is able to establish in contaminated environments, which may be associated to its ability to accumulate melanin that protects against environmental stressors (Kõljalg et al., 2000; Thiem et al., 2018).

5.4.4. Dominant role of saprotrophic and mycorrhizal functional guilds

Plant composition affect the richness and the relative abundance of saprotrophic, pathogenic and mycorrhizal fungi in soils (Gao et al., 2017; Saitta et al., 2018a). The highest abundance of ECM fungi in white poplar and stone pine soils is very likely to be related to the symbiosis with these fungi. Moreover, these soils registered a reduction of other guilds such as plant pathogens (Wang et al., 2019) and saprotrophs (Fernandez and Kennedy, 2016). Pathogenic and mycorrhizal fungi are more dependent on the presence of particular host species due to their biotrophy (Saitta et al., 2018a). The relative abundance of mycorrhizal fungi was certainly dependent on the ECM host species, but the relative abundance of pathogenic fungi may be outcompeted by ECM fungi due to their ability to defend their host against plant pathogens (Wang et al., 2019). In fact, the pathogen guild was more abundant in non-remediated bare soils without vegetation. Maybe in these non-remediated soils, plant communities were unable to establish due to the joint effect of toxic levels of trace elements and the abundance of pathogenic fungi. However, taxonomical identification of pathogenic fungi cannot ensure their pathogenicity (Wang et al., 2019).

Saprotrophs were abundant in all habitat types but especially in non-ECM hosts (grassland and wild olive soils). The decline in the abundance of saprotrophs in soils with ECM hosts may be explained by the increase of ECM fungi, as exposed above. This suppression of saprotrophs under white poplar and stone pine soils might also contribute to the accumulation of organic matter in the soils underneath these species (Sterkenburg et al., 2018). Saprotrophic fungi and other soil microbes play an important role in organic matter decomposition and nutrient cycling through their secretion of extracellular enzyme activities (Talbot et al., 2013). Dehydrogenase activity was very low in the non-remediated soils possibly due to acidic pH, very low N and organic C content, and the high S and As concentrations. In vegetated soils, dehydrogenase activity increased, likely due to the more favourable pH conditions and the higher N availability and sandy texture. Although dehydrogenase activity is closely correlated with soil respiration (Lee et al.,

2002), in this study soil respiration was not significantly different among habitats. We must note, however, that respiration in the non-remediated soils was very low, under the detection limit.

Among functional guilds, mycorrhizal fungi are a major group as they perform a crucial function on plant development and in soil genesis, modulating interactions between plants and soils (Dickie et al., 2013). The most common mycorrhizas are AM which associate with ca. 74% of Angiosperm species while ECM associate with ca. 2% species (Brundrett, 2009; van der Heijden et al., 2015). However, the contribution of AM fungal taxa to mycorrhizal species richness in our study was low (11%) in comparison to ECM fungi (79%), probably due to their reduced taxonomic diversity (ca. 300 known species) compared to ECM guild (20,000-25,000 species) (Öpik et al., 2010; Rinaldi et al., 2008). We expected AM fungi to be more abundant and diverse in soils covered by AM plant hosts (Davison et al., 2015; Rillig, 2004). However, the general low abundance of the AM fungal phylum (Glomeromycota) in the whole study probably impeded to find wide differences in their diversity and abundance between pure plant AM hosts (wild olive and grasslands) and the ECM-AM dual hosts (stone pine and white poplar) (Dickie et al., 2013; Horton et al., 1998). Arbuscular mycorrhizal fungi was also reduced due to the contamination negative effect (Cu, Fe and Mn) which may be explained by the lower tolerance of AM fungi to chemical stress, in comparison to ECM fungi (Tedersoo and Bahram, 2019).

Ectomycorrhizal fungi are in symbiosis with many dominant trees in forest ecosystems favouring host survival by improving nutrient and water uptake (Tedersoo and Bahram, 2019). In contaminated forests, ECM fungi alleviate trace element toxicity although species composition varied along the contamination gradient (Huang et al., 2014). Among the most common ECM fungal taxa, *Thelephoraceae*, *Inocybaceae* and *Pyronemataceae* were well represented, as found in other studies (Horton and Bruns, 2001; Kõljalg et al., 2000). However, common *Russulaceae*, *Cortinariaceae* and *Sebacinaceae* taxa were rare in the study site. A common ECM species, *Cenococcum geophilum* was absent in our samples. The genera *Tomentella* was the most widespread ECM fungi among studied habitats, as found in other areas (Horton and Bruns, 2001; Kõljalg et al., 2000) even in contaminated ones (Huang et al., 2014). Although the genera *Tuber* may be limited by acidic pH in forest soils (Richard et al., 2005), *Tuber* was present in a variety of soils, including the acidic soils.

5.4.5. Implications for management

Twenty years after a mine spill that contaminated the study area, highly contaminated areas with acidic pH and low nutrient content were still present. In soils where phytoremediation was not applied, vegetation was not able to naturally establish, which likely contributed to the lower fungal diversity and relative abundance. The community in these soils was dominated by pathogenic fungi, which possibly contributed to the limited plant establishment. For the soils where phytoremediation was applied, the strategy adopted promoted a higher abundance diversity of fungal communities in comparison to non-remediated soils. The first intervention, sludge removal and application of different amendments, proved to be effective for the establishment of herbaceous communities. These grassland habitats support high values of fungal diversity, richness, taxonomy and functionality compared to non-remediated soils. The second intervention, planting different tree species, allowed the establishment of different habitats for fungi, further recruiting more fungal taxa, mainly within the ECM fungal guild. The selection of tree species in phytoremediated areas should take the multiple effects of these species in the ecosystem into account. In this study, white poplar seemed to be the best tree species to plant due to its positive effect on soil properties (organic matter and N accumulation), and its reduced acidifying effect on soil, in comparison to pine trees. These positive effects on soils were associated to a higher richness and diversity of fungal communities. However, other side effects of the plantation of this tree species in contaminated areas should be further studied, such as the capacity of accumulating contaminants in aerial biomass (Domínguez et al., 2008). Moreover, the studied ecosystem benefits from the variety of habitats and species established in the Guadiamar Green Corridor, as each habitat offers a refuge to diverse soil fungal community and structure.

5.5. Conclusions

In this study, we demonstrated the importance of the plant species identity in the long-term establishment of fungal species in degraded areas exposed to phytoremediation strategies. Plant cover was the main driver of the composition and structure of soil fungal

communities. Life history of the planted tree species seemed to be decisive to functional guilds of soil fungi, with ECM-host trees being key in recruiting ECM fungi. Afforestation with different tree species showed species-specific effects on soil nutrient and C contents which may have led to increased spatial heterogeneity in the areas with a potential to recruit a wider diversity of fungi.

5.6 Bibliography

Amend, A., 2014. From dandruff to deep-sea vents: *Malassezia*-like fungi are ecologically hyper-diverse. PLoS Pathogens 10, e1004277.

Banning, N.C., Gleeson, D.B., Grigg, A.H., Grant, C.D., Andersen, G.L., Brodie, E.L., Murphy, D. V., 2011. Soil microbial community successional patterns during forest ecosystem restoration. Applied and Environmental Microbiology 77, 6158–6164.

Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting linear mixed-effects models using lme4. Journal of Statistical Software 67, 1–48.

Bellemain, E., Carlsen, T., Brochmann, C., Coissac, E., Taberlet, P., Kauserud, H., 2010. ITS as an environmental DNA barcode for fungi: an in silico approach reveals potential PCR biases. BMC Microbiology 10, 1–9.

Berg, G., Smalla, K., 2009. Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. FEMS Microbiology Ecology 68, 1–13.

Bolan, N.S., Park, J.H., Robinson, B., Naidu, R., Huh, K.Y., 2011. Phytostabilization: a green approach to contaminant containment. Advances in Agronomy 112, 145–204.

Buée, M., Reich, M., Murat, C., Morin, E., Nilsson, R.H., Uroz, S., Martin, F., 2009. 454 Pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. New Phytologist 184, 449–456.

Bünemann, E.K., Bongiorno, G., Bai, Z., Creamer, R.E., De Deyn, G., de Goede, R., Fleskens, L., Geissen, V., Kuyper, T.W., Mäder, P., Pulleman, M., Sukkel, W., van Groenigen, J.W., Brussaard, L., 2018. Soil quality – A critical review. Soil Biology and Biochemistry 120, 105–125.

Burgos, P., Madejón, P., Madejón, E., Girón, I., Cabrera, F., Murillo, J.M., 2013. Natural remediation of an unremediated soil twelve years after a mine accident: trace element mobility and plant composition. Journal of Environmental Management 114, 36–45.

Cabral, L., Soares, C.R.F.S., Giachini, A.J., Siqueira, J.O., 2015. Arbuscular mycorrhizal fungi in phytoremediation of contaminated areas by trace elements: mechanisms and major benefits of their applications. World Journal of Microbiology and Biotechnology 31, 1655–1664.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high resolution sample inference from Illumina amplicon data. Nat Methods 13, 581–583.

Campbell, C.D., Chapman, S.J., Cameron, C.M., Davidson, M.S., Potts, J.M., 2003. A rapid microtiter plate method to measure carbon dioxide evolved from carbon substrate

amendments so as to determine the physiological profiles of soil microbial communities by using whole soil. Applied and Environmental Microbiology 69, 3593–3599.

Chaparro, J.M., Badri, D. V., Vivanco, J.M., 2014. Rhizosphere microbiome assemblage is affected by plant development. ISME Journal 8, 790–803.

Davison, J., Moora, M., Öpik, M., Adholeya, A., Ainsaar, L., Bâ, A., Burla, S., Diedhiou, A.G., Hiiesalu, I., Jairus, T., Johnson, N.C., Kane, A., Koorem, K., Kochar, M., Ndiaye, C., Pärtel, M., Reier, Ü., Saks, Ü., Singh, R., Vasar, M., Zobel, M., 2015. Global assessment of arbuscular mycorrhizal fungus diversity reveals very low endemism. Science 349, 970–973.

De Cáceres, M., Legendre, P., 2009. Associations between species and groups of sites: indices and statistical inference. Ecology 90, 3566–3574.

de Vries, F.T., Manning, P., Tallowin, J.R.B., Mortimer, S.R., Pilgrim, E.S., Harrison, K.A., Hobbs, P.J., Quirk, H., Shipley, B., Cornelissen, J.H.C., Kattge, J., Bardgett, R.D., 2012. Abiotic drivers and plant traits explain landscape-scale patterns in soil microbial communities. Ecology Letters 15, 1230–1239.

Demolon, A., Leroux, D., 1954. Guide pour l´étude expérimentale du sol. Soil Science 74, 405.

Dickie, I.A., Koele, N., Blum, J.D., Gleason, J.D., McGlone, M.S., 2014. Mycorrhizas in changing ecosystems. Botany 92, 149-160.

Dickie, I.A., Martínez-García, L.B., Koele, N., Grelet, G.A., Tylianakis, J.M., Peltzer, D.A., Richardson, S.J., 2013. Mycorrhizas and mycorrhizal fungal communities throughout ecosystem development. Plant and Soil 367, 11–39.

Dini-Andreote, F., Stegen, J.C., Van Elsas, J.D., Salles, J.F., 2015. Disentangling mechanisms that mediate the balance between stochastic and deterministic processes in microbial succession. Proceedings of the National Academy of Sciences of the United States of America 112, E1326–E1332.

Djemiel, C., Grec, S., Hawkins, S., 2017. Characterization of bacterial and fungal community dynamics by high-throughput sequencing (HTS) metabarcoding during flax dew-retting. Frontiers in Microbiology 8, 1–16.

Domínguez, M.T., Alegre, J.M., Madejón, P., Madejón, E., Burgos, P., Cabrera, F., Marañón, T., Murillo, J.M., 2016. River banks and channels as hotspots of soil pollution after large-scale remediation of a river basin. Geoderma 261, 133–140.

Domínguez, M.T., Marañón, T., Murillo, J.M., Schulin, R., Robinson, B.H., 2010. Nutritional status of Mediterranean trees growing in a contaminated and remediated area. Water, Air, and Soil Pollution 205, 305–321.

Domínguez, M.T., Marañón, T., Murillo, J.M., Schulin, R., Robinson, B.H., 2008. Trace element accumulation in woody plants of the Guadiamar Valley, SW Spain: a large-scale phytomanagement case study. Environmental Pollution 152, 50–59.

Dufrêne, M., Legendre, P., 1997. Species assemblages and indicator species: the need for a flexible asymmetrical approach. Ecological Monographs 67, 345–366.

Fernandez, C.W., Kennedy, P.G., 2016. Revisiting the 'Gadgil effect': do interguild fungal interactions control carbon cycling in forest soils? New Phytologist 209, 1382–1394.

Finzi, A.C., Van Breemen, N., Canham, C.D., 1998. Canopy tree-soil interactions within temperate forests: species effects on pH and cations. Ecological Applicaitons 8, 440-446.

Foulon, J., Zappelini, C., Durand, A., Valot, B., Girardclos, O., Blaudez, D., Chalot, M., 2016. Environmental metabarcoding reveals contrasting microbial communities at two poplar phytomanagement sites. Science of the Total Environment 571, 1230–1240.

Frøslev, T.G., Kjøller, R., Bruun, H.H., Ejrnæs, R., Brunbjerg, A.K., Pietroni, C., Hansen, A.J., 2017. Algorithm for post-clustering curation of DNA amplicon data yields reliable biodiversity estimates. Nature Communications 8, 1188.

Gao, C., Shi, N.N., Chen, L., Ji, N.N., Wu, B.W., Wang, Y.L., Xu, Y., Zheng, Y., Mi, X.C., Ma, K.P., Guo, L.D., 2017. Relationships between soil fungal and woody plant assemblages differ between ridge and valley habitats in a subtropical mountain forest. New Phytologist 213, 1874–1885.

García-Carmona, M., García-Robles, H., Turpín Torrano, C., Fernández Ondoño, E., Lorite Moreno, J., Sierra Aragón, M., Martín Peinado, F.J., 2019. Residual pollution and vegetation distribution in amended soils 20 years after a pyrite mine tailings spill (Aznalcóllar, Spain). Science of the Total Environment 650, 933–940.

Gardes, M., Bruns, T.. D., 1993. ITS primers with enhanced specificity for basidiomycetes—Application to identification of mycorhizae and rusts. Molecular Ecology 2, 113–8.

Gardi, C., Jeffery, S., Saltelli, A., 2013. An estimate of potential threats levels to soil biodiversity in EU. Global Change Biology 19, 1538–1548.

Gee, G.W., Bauder, J.W., 1979. Particle size analysis by hydrometer: a simplified method for routine textural analysis and a sensitivity test of measurement parameters. Soil Science Society American Journal 43, 1004–1007.

Geisen, S., Wall, D.H., van der Putten, W.H., 2019. Challenges and opportunities for soil biodiversity in the Anthropocene. Current Biology 29, R1036–R1044.

Horton, T.R., Bruns, T.D., 2001. The molecular revolution in ectomycorrhizal ecology: Peeking into the black-box. Molecular Ecology 10, 1855–1871.

Horton, T.R., Cázares, E., Bruns, T.D., 1998. Ectomycorrhizal, vesicular-arbuscular and dark septate fungal colonization of bishop pine (*Pinus muricata*) seedlings in the first 5 months of growth after wildfire. Mycorrhiza 8, 11–18.

Huang, J., Nara, K., Zong, K., Wang, J., Xue, S., Peng, K., Shen, Z., Lian, C., 2014. Ectomycorrhizal fungal communities associated with Masson pine (*Pinus massoniana*) and white oak (*Quercus fabri*) in a manganese mining region in Hunan Province, China. Fungal Ecology 9, 1–10.

Hujslová, M., Gryndler, M., 2019. Fungi in biofilms of highly acidic soils, in: Tiquia-Arashiro, S.M., Grube, M. (Eds.), Fungi in Extreme Environments: Ecological Role and Biotechnological Significance. Springer Nature Switzerland, Cham, pp. 185–203.

Hujslová, M., Kubátová, A., Kostovčík, M., Kolařík, M., 2013. *Acidiella bohemica* gen. et sp. nov. and *Acidomyces* spp. (Teratosphaeriaceae), the indigenous inhabitants of extremely acidic soils in Europe. Fungal Diversity 58, 33–45.

Kõljalg, U., Dahlberg, A., Taylor, A.F.S., Larsson, E., Hallenberg, N., Stenlid, J., Larsson, K.H., Fransson, P.M., Kårén, O., Jonsson, L., 2000. Diversity and abundance of resupinate thelephoroid fungi as ectomycorrhizal symbionts in Swedish boreal forests. Molecular Ecology 9, 1985–1996.

Kõljalg, U., Nilsson, R.H., Abarenkov, K., Tedersoo, L., Taylor, A.F.S., Bahram, M., Bates, S.T., 2013. Towards a unified paradigm for sequence-based identification of fungi. Molecular Ecology 22, 5271–5277.

Kraus, U., Wiegand, J., 2006. Long-term effects of the Aznalcóllar mine spill-heavy metal content and mobility in soils and sediments of the Guadiamar river valley (SW Spain). Science of the Total Environment 367, 855–871.

Lee, I.S., Kim, O.K., Chang, Y.Y., Bae, B., Kim, H.H., Baek, K.H., 2002. Heavy metal concentrations and enzyme activities in soil from a contaminated Korean shooting range. Journal of Bioscience and Bioengineering 94, 406–411.

Leff, J.W., Bardgett, R.D., Wilkinson, A., Jackson, B.G., Pritchard, W.J., De Long, J.R., Oakley, S., Mason, K.E., Ostle, N.J., Johnson, D., Baggs, E.M., Fierer, N., 2018. Predicting the structure of soil communities from plant community taxonomy, phylogeny, and traits. ISME Journal 12, 1794–1805.

Madejón, P., Domínguez, M.T., Gil-Martínez, M., Navarro-Fernández, C.M., Montiel-Rozas, M.M., Madejón, E., Murillo, J.M., Cabrera, F., Marañón, T., 2018. Evaluation of amendment addition and tree planting as measures to remediate contaminated soils: the Guadiamar case study (SW Spain). Catena 166, 34–43.

McMurdie, P.J., Holmes, S., 2014. Waste not, want not: why rarefying microbiome data is inadmissible. PLoS Computational Biology 10, e1003531.

McMurdie, P.J., Holmes, S., 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS ONE 8, e61217.

Meiser, A., Bálint, M., Schmitt, I., 2014. Meta-analysis of deep-sequenced fungal communities indicates limited taxon sharing between studies and the presence of biogeographic patterns. New Phytologist 201, 623–635.

Montiel-Rozas, M. del M., López-García, Á., Kjøller, R., Madejón, E., Rosendahl, S., 2016. Organic amendments increase phylogenetic diversity of arbuscular mycorrhizal fungi in acid soil contaminated by trace elements. Mycorrhiza 26, 575–585.

Mueller, R.C., Paula, F.S., Mirza, B.S., Rodrigues, J.L.M., Nüsslein, K., Bohannan, B.J.M., 2014. Links between plant and fungal communities across a deforestation chronosequence in the Amazon rainforest. ISME Journal 8, 1548–1550.

Narendrula-Kotha, R., Nkongolo, K.K., 2017. Bacterial and fungal community structure and diversity in a mining region under long-term metal exposure revealed by metagenomics sequencing. Ecological Genetics and Genomics 2, 13–24.

Nguyen, N.H., Song, Z., Bates, S.T., Branco, S., Tedersoo, L., Menke, J., Schilling, J.S., Kennedy, P.G., 2016. FUNGuild: an open annotation tool for parsing fungal community datasets by ecological guild. Fungal Ecology 20, 241–248.

Ni, Y., Yang, T., Zhang, K., Shen, C., Chu, H., 2018. Fungal communities along a small-scale elevational gradient in an alpine tundra are determined by soil carbon nitrogen ratios. Frontiers in Microbiology 9, 1815.

Nilsson, R.H., Anslan, S., Bahram, M., Wurzbacher, C., Baldrian, P., Tedersoo, L., 2019. Mycobiome diversity: high-throughput sequencing and identification of fungi. Nature Reviews Microbiology 17, 95–109.

O'Brien, H.E., Parrent, J.L., Jackson, J.A., Moncalvo, J.M., Vilgalys, R., 2005. Fungal community analysis by large-scale sequencing of environmental samples. Applied and Environmental Microbiology 71, 5544–5550.

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., 2016. Vegan: Community Ecology Package.

Op De Beeck, M., Lievens, B., Busschaert, P., Rineau, F., Smits, M., Vangronsveld, J., Colpaert, J. V., 2015. Impact of metal pollution on fungal diversity and community structures. Environmental Microbiology 17, 2035–2047.

Peay, K.G., Baraloto, C., Fine, P.V.A., 2013. Strong coupling of plant and fungal community structure across western Amazonian rainforests. ISME Journal 7, 1852–1861.

Peršoh, D., 2015. Plant-associated fungal communities in the light of meta'omics. Fungal Diversity 75, 1–25.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., R Core Team, 2016. nlme: linear and nonlinear mixed effects models.

Pulford, I.D., Watson, C., 2003. Phytoremediation of heavy metal-contaminated land by trees - A review. Environment International 29, 529–540.

Richard, F., Millot, S., Gardes, M., Selosse, M.A., 2005. Diversity and specificity of ectomycorrhizal fungi retrieved from an old-growth Mediterranean forest dominated by Quercus ilex. New Phytologist 166, 1011–1023.

Richardson, D. (Ed.), 2000. Ecology and biogeography of Pinus. Cambridge University Press.

Rillig, M.C., 2004. Arbuscular mycorrhizae and terrestrial ecosystem processes. Ecology Letters 7, 740–754.

Rincón, A., Santamaría-Pérez, B., Rabasa, S.G., Coince, A., Marçais, B., Buée, M., 2015. Compartmentalized and contrasted response of ectomycorrhizal and soil fungal communities of Scots pine forests along elevation gradients in France and Spain. Environmental Microbiology 17, 3009–3024.

Rincón, A., Santamaría-Pérez, B., Rabasa, S.G., Coince, A., Marçais, B., Buée, M., 2015. Compartmentalized and contrasted response of ectomycorrhizal and soil fungal communities of Scots pine forests along elevation gradients in France and Spain. Environmental Microbiology 17, 3009–3024.

Rodríguez-Eugenio, N., McLaughlin, M., Pennock, D., 2018. Soil pollution: a hidden reality. FAO, Rome.

Rognes, T., Flouri, T., Nichols, B., Quince, C., Mahé, F., 2016. VSEARCH: a versatile open source tool for metagenomics. PeerJ 4, e2584.

Rubin, B.E.R., Gibbons, S.M., Kennedy, S., Hampton-Marcell, J., Owens, S., Gilbert, J.A., 2013. Investigating the impact of storage conditions on microbial community composition in soil samples. PLoS ONE 8, e70460.

Saitta, A., Anslan, S., Bahram, M., Brocca, L., Tedersoo, L., 2018. Tree species identity and diversity drive fungal richness and community composition along an elevational gradient in a Mediterranean ecosystem. Mycorrhiza 28, 39–47.

Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque, C.A., Chen, W., Bolchacova, E., Voigt, K., Crous, P.W., Miller, A.N., Wingfield, M.J., Aime, M.C., An, K.D., Bai, F.Y., Barreto, R.W., Begerow, D., Bergeron, M.J., Blackwell, M., Boekhout, T., Bogale, M., Boonyuen, N., Burgaz, A.R., Buyck, B., Cai, L., Cai, Q., Cardinali, G., Chaverri, P., Coppins, B.J., Crespo, A., Cubas, P., Cummings, C., Damm, U., de Beer, Z.W., de Hoog, G.S., Del-Prado, R., Dentinger, B., Diéguez-Uribeondo, J., Divakar, P.K., Douglas, B., Dueñas, M., Duong, T.A., Eberhardt, U., Edwards, J.E., Elshahed, M.S., Fliegerova, K., Furtado, M., García, M.A., Ge, Z.W., Griffith, G.W., Griffiths, K., Groenewald, J.Z., Groenewald, M., Grube, M., Gryzenhout, M., Guo, L.D., Hagen, F., Hambleton, S., Hamelin, R.C., Hansen, K., Harrold, P., Heller, G., Herrera, C., Hirayama, K., Hirooka, Y., Ho, H.M., Hoffmann, K., Hofstetter, V., Högnabba, F., Hollingsworth, P.M., Hong, S.B., Hosaka, K., Houbraken, J., Hughes, K., Huhtinen, S., Hyde, K.D., James, T., Johnson, E.M., Johnson, J.E., Johnston, P.R., Jones, E.B.G., Kelly, L.J., Kirk, P.M., Knapp, D.G., Kõljalg, U., Kovács, G.M., Kurtzman, C.P., Landvik, S., Leavitt, S.D., Liggenstoffer, A.S., Liimatainen, K., Lombard, L., Luangsa-ard, J.J., Lumbsch, H.T., Maganti, H., Maharachchikumbura, S.S.N., Martin, M.P., May, T.W., McTaggart, A.R., Methven, A.S., Meyer, W., Moncalvo, J.M., Mongkolsamrit, S., Nagy, L.G., Nilsson, R.H., Niskanen, T., Nyilasi, I., Okada, G., Okane, I., Olariaga, I., Otte, J., Papp, T., Park, D., Petkovits, T., Pino-Bodas, R., Quaedvlieg, W., Raja, H.A., Redecker, D., Rintoul, T.L., Ruibal, C., Sarmiento-Ramírez, J.M., Schmitt, I., Schüßler, A., Shearer, C., Sotome, K., Stefani, F.O.P., Stenroos, S., Stielow, B., Stockinger, H., Suetrong, S., Suh, S.O., Sung, G.H., Suzuki, M., Tanaka, K., Tedersoo, L., Telleria, M.T., Tretter, E., Untereiner, W.A., Urbina, H., Vágvölgyi, C., Vialle, A., Vu, T.D., Walther, G., Wang, Q.M., Wang, Y., Weir, B.S., Weiß, M., White, M.M., Xu, J., Yahr, R., Yang, Z.L., Yurkov, A., Zamora, J.C., Zhang,

N., Zhuang, W.Y., Schindel, D., 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proceedings of the National Academy of Sciences of the United States of America 109, 6241–6246.

Shakya, M., Gottel, N., Castro, H., Yang, Z.K., Gunter, L., Labbé, J., Muchero, W., Bonito, G., Vilgalys, R., Tuskan, G., Podar, M., Schadt, C.W., 2013. A multifactor analysis of fungal and bacterial community structure in the root microbiome of mature *Populus deltoides* trees. PLoS ONE 8, e76382.

Smith, S., Read, D., 2008. Mycorrhizal Symbiosis, 3rd ed. Academic Press, London.

Sterkenburg, E., Clemmensen, K.E., Ekblad, A., Finlay, R.D., 2018. Contrasting effects of ectomycorrhizal fungi on early and late stage decomposition in a boreal forest. The ISME Journal 12, 2187–2197.

Sun, H., Terhonen, E., Kovalchuk, A., Tuovila, H., Chen, H., Oghenekaro, A.O., Heinonsalo, J., Kohler, A., Kasanen, R., Vasander, H., Asiegbu, F.O., 2016. Dominant tree species and soil type affect the fungal community structure in a boreal peatland forest. Applied and Environmental Microbiology 82, 2632–2643.

Sun, S., Li, S., Avera, B.N., Strahm, B.D., Badgley, B.D., 2017. Soil bacterial and fungal communities show distinct recovery patterns during forest ecosystem restoration. Applied and Environmental Microbiology 83, 1–14.

Talbot, J.M., Bruns, T.D., Smith, D.P., Branco, S., Glassman, S.I., Erlandson, S., Vilgalys, R., Peay, K.G., 2013. Independent roles of ectomycorrhizal and saprotrophic communities in soil organic matter decomposition. Soil Biology and Biochemistry 57, 282–291.

Tedersoo, L., Bahram, M., 2019. Mycorrhizal types differ in ecophysiology and alter plant nutrition and soil processes. Biological Reviews 1868, 1857–1880.

Tedersoo, L., Bahram, M., Dickie, I.A., 2014a. Does host plant richness explain diversity of ectomycorrhizal fungi? Re-evaluation of Gao *et al.* (2013) data sets reveals sampling effects. Molecular Ecology 23, 992–995.

Tedersoo, L., Bahram, M., Põlme, S., Kõljalg, U., Yorou, N.S., Wijesundera, R., Ruiz, L.V., Vasco-Palacios, A.M., Thu, P.Q., Suija, A., Smith, M.E., Sharp, C., Saluveer, E., Saitta, A., Rosas, M., Riit, T., Ratkowsky, D., Pritsch, K., Põldmaa, K., Piepenbring, M., Phosri, C., Peterson, M., Parts, K., Pärtel, K., Otsing, E., Nouhra, E., Njouonkou, A.L., Nilsson, R.H., Morgado, L.N., Mayor, J., May, T.W., Majukim, L., Lodge, D.J., Lee, S.S., Larsson, K.-H., Kohout, P., Hosaka, K., Hiiesalu, I., Henkel, T.W., Harend, H., Guo, L., Greslebin, A., Gretlet, G., Geml, J., Gates, G., Dunstan, W., Dunk, C., Drenkhan, R., Dearnaley, J., Kesel, A. De, Dang, T., Chen, X., Buegger, F., Brearley, F.Q., Bonito, G., Anslan, S., Abell, S., Abarenkov, K., 2014b. Global diversity and geography of soil fungi. Science 346, 1256688.

Tedersoo, L., Bahram, M., Toots, M., Diédhiou, A.G., Henkel, T.W., Kjoller, R., Morris, M.H., Nara, K., Nouhra, E., Peay, K.G., Põlme, S., Ryberg, M., Smith, M.E., Kõljalg, U., 2012. Towards global patterns in the diversity and community structure of ectomycorrhizal fungi. Molecular Ecology 21, 4160–4170.

Tedersoo, L., May, T.W., Smith, M.E., 2010. Ectomycorrhizal lifestyle in fungi: Global diversity, distribution, and evolution of phylogenetic lineages. Mycorrhiza 20, 217–263.

Trevors, J.T., 1984. Dehydrogenase activity in soil: a comparison between the INT and TTC assay. Soil Biology and Biochemistry 16, 673–674.

UNITE Community, 2017. UNITE general FASTA release.

Unterseher, M., Schnittler, M., Dormann, C., Sickert, A., 2008. Application of species richness estimators for the assessment of fungal diversity. FEMS Microbiology Letters 282, 205–213.

Urbanová, M., Šnajdr, J., Baldrian, P., 2015. Composition of fungal and bacterial communities in forest litter and soil is largely determined by dominant trees. Soil Biology and Biochemistry 84, 53–64.

van der Heijden, M.G.A., Bardgett, R.D., Van Straalen, N.M., 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecology Letters 11, 296–310.

van der Heijden, M.G.A., Klironomos, J.N., Ursic, M., Moutoglis, P., Streitwolf-Engel, R., Boller, T., Wiemken, A., Sanders, I.R., 1998. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature 396, 69–72.

van der Linde, S., Suz, L.M., Orme, C.D.L., Cox, F., Andreae, H., Asi, E., Atkinson, B., Benham, S., Carroll, C., Cools, N., De Vos, B., Dietrich, H.-P., Eichhorn, J., Gehrmann, J., Grebenc, T., Gweon, H.S., Hansen, K., Jacob, F., Kristöfel, F., Lech, P., Manninger, M., Martin, J., Meesenburg, H., Merilä, P., Nicolas, M., Pavlenda, P., Rautio, P., Schaub, M., Schröck, H.-W., Seidling, W., Šrámek, V., Thimonier, A., Thomsen, I.M., Titeux, H., Vanguelova, E., Verstraeten, A., Vesterdal, L., Waldner, P., Wijk, S., Zhang, Y., Žlindra, D., Bidartondo, M.I., 2018. Environment and host as large-scale controls of ectomycorrhizal fungi. Nature 258, 243-250.

Vázquez-Campos, X., Kinsela, A.S., Waite, T.D., Collins, R.N., Neilan, B.A., 2014. *Fodinomyces uranophilus* gen. nov. sp. nov. and *Coniochaeta fodinicola* sp. nov., two uranium mine-inhabiting Ascomycota fungi from northern Australia. Mycologia 106, 1073–1089.

Veresoglou, S.D., Halley, J.M., Rillig, M.C., 2015. Extinction risk of soil biota. Nature Communications 6, 1–10.

Voříšková, J., Baldrian, P., 2013. Fungal community on decomposing leaf litter undergoes rapid successional changes. ISME Journal 7, 477–486.

Wang, L., Ji, B., Hu, Y., Liu, R., Sun, W., 2017. A review on *in situ* phytoremediation of mine tailings. Chemosphere 184, 594–600.

Wang, P., Chen, Y., Sun, Y., Tan, S., Zhang, S., Wang, Z., Zhou, J., Zhang, G., Shu, W., Luo, C., Kuang, J., 2019. Distinct biogeography of different fungal guilds and their associations with plant species richness in forest ecosystems. Frontiers in Ecology and Evolution 7, 1–13.

White, T.J., Bruns, T., Lee, S., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, in: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, Inc., New York.

Wickham, H., 2016. ggplot2: elegant graphics for data analysis. Springer-Verlag, New York.

Wurzburger, N., Hendrick, R.L., 2007. Rhododendron thickets alter N cycling and soil extracellular enzyme activities in southern Appalachian hardwood forests. Pedobiologia 50, 563-576.

Zappelini, C., Karimi, B., Foulon, J., Lacercat-Didier, L., Maillard, F., Valot, B., Blaudez, D., Cazaux, D., Gilbert, D., Yergeau, E., Greer, C., Chalot, M., 2015. Diversity and complexity of microbial communities from a chlor-alkali tailings dump. Soil Biology and Biochemistry 90, 101–110.

5.6 Supplementary material

A

B

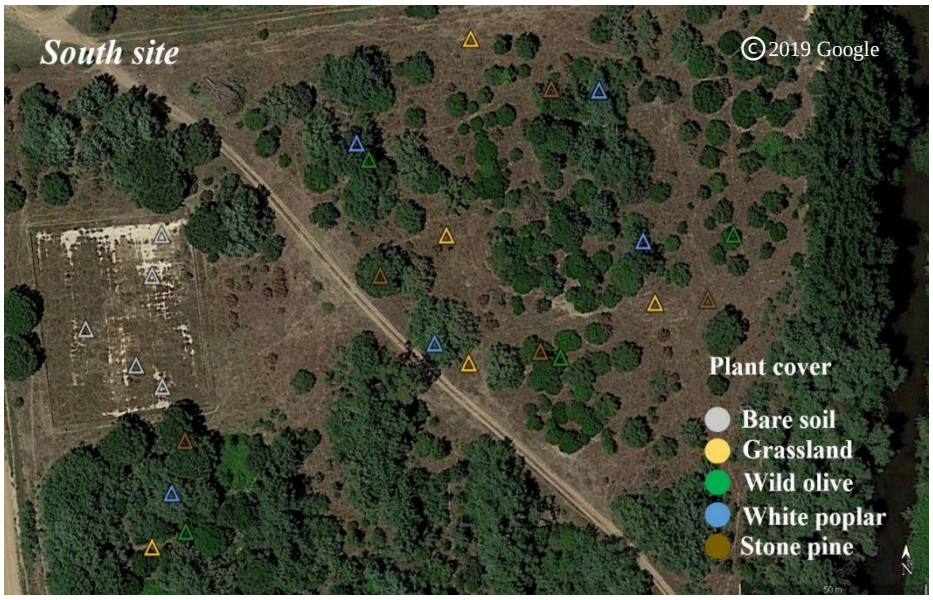


Figure S5.1 Location map of the study area. A) Guadiamar Green Corridor and studied sites. B) North site sampled points. C) South site sampled points. Map images from Junta de Andalucía (Consejería de Agricultura, Ganadería, Pesca y Desarrollo Sostenible), OpenStreetMap (https://www.openstreetmap.org/copyright) and Google Earth, version 7.3.2.5776 (earth.google.com/web/).

Table S5.1 Tree height and crown projection of tree species specimens.

	Height (m)		Crown projection (m^2)	
Tree species	North	South	North	South
Olea europaea	3.3	4.1	8.8	11.8
	3.5	4.2	9.7	20.2
	3.5	5.8	6.6	26.5
	3.7	6.2	18.4	39.9
	3.9	6.8	5.7	59.8
Populus alba	8.9	8.0	45.5	25.1
	10.7	9.2	84.9	34.2
	11.7	9.6	63.5	30.8
	13.9	10.0	53.6	102.5
	17.7	10.8	102.9	104.0
Pinus pinea	8.1	7.6	27.1	32.2
	8.3	7.8	35.2	22.2
	8.7	8.9	52.0	64.3
	9.5	9.4	44.8	86.3
	9.5	9.8	51.7	110.3

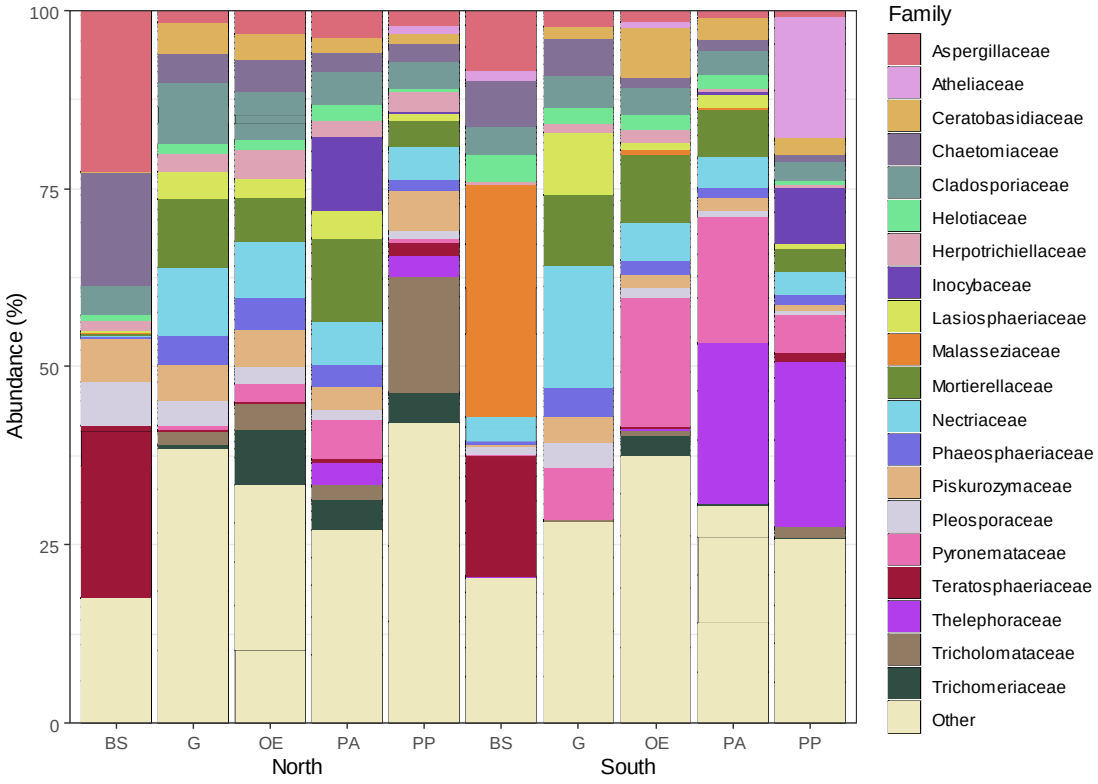


Figure S5.2 Relative abundance of top 20 most abundant fungal families in the 10 habitat types according to plant cover (BS = Bare soil, G = Grassland, OE = *Olea europaea*, PA = *Populus alba*, PP = *Pinus pinea*) and site (North, South) factors.

Table S5.2 Indicator species analyses. A) Number of OTUs associated to the plant cover and site factors. B) Fungal species found in South site bare soils.

A

	Number of OTUs	
Plant cover	North	South
Bare soil	96	11
Grassland	90	374
Olea europaea	50	28
Populus alba	165	246
Pinus pinea	150	151

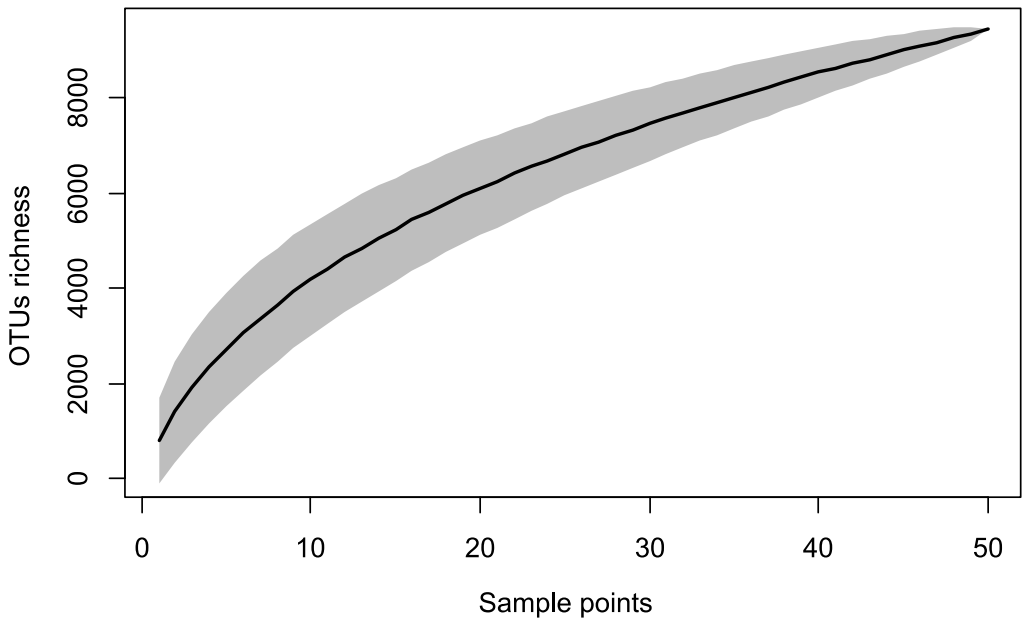
B

Indicator species of non-remediated bare soils in South site
Coniochaeta fodinicola
Fodinomyces uranophilus
Malassezia globosa
Malassezia restricta
Pyrenopeziza revincta
Rachicladosporium sp.
Sarocladium kiliense

Table S5.3 Number of total OTUs and percentage of γ-diversity in North and South soils, according to the plant cover factor. Shared OTUs between the two sites are indicated for each plant cover.

	North	South	Shared
Plant cover	Total (%)	Total (%)	Total (%)
Bare soil	926 (14.7)	168 (3.0)	87 (8.6)
Grassland	2603 (41.3)	2735 (48.2)	944 (21.5)
Olea europaea	1953 (31.0)	1845 (32.5)	630 (19.9)
Populus alba	3021 (47.9)	2919 (51.5)	1251 (26.7)
Pinus pinea	2886 (45.8)	2850 (50.3)	1063 (22.7)
γ-diversity	6301 (100.0)	5669 (100.0)	

A



B

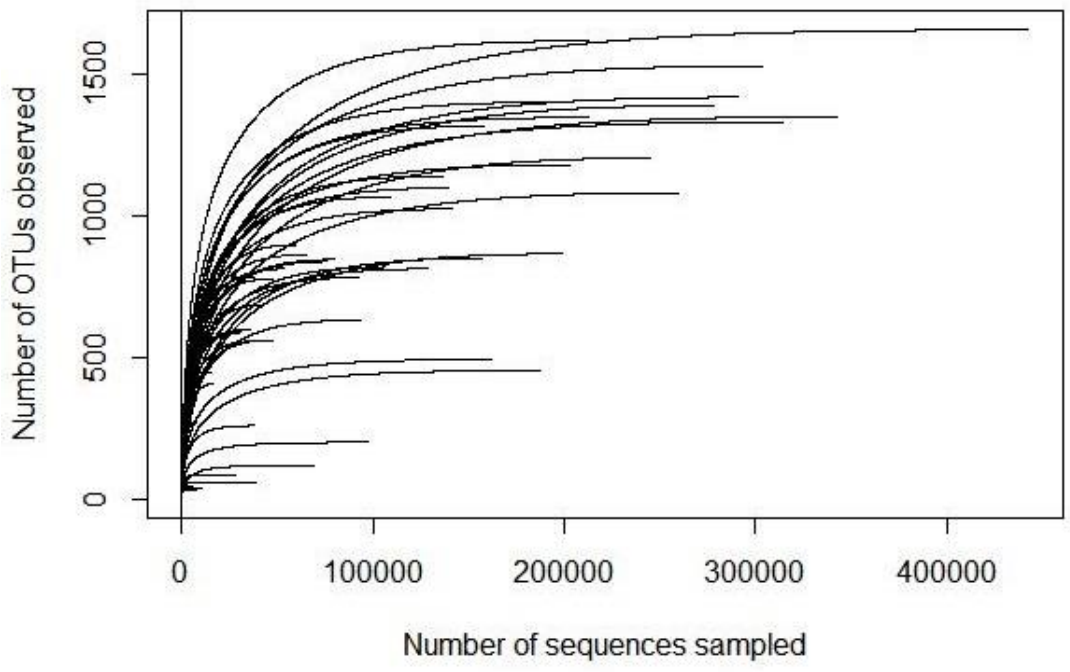


Figure S5.3 A) Species accumulation curve with total species richness in all sample points (n = 50). Confidence interval (0.95) in grey. B) Sampling effort curves for the fungal community in the 50 soils sampled.

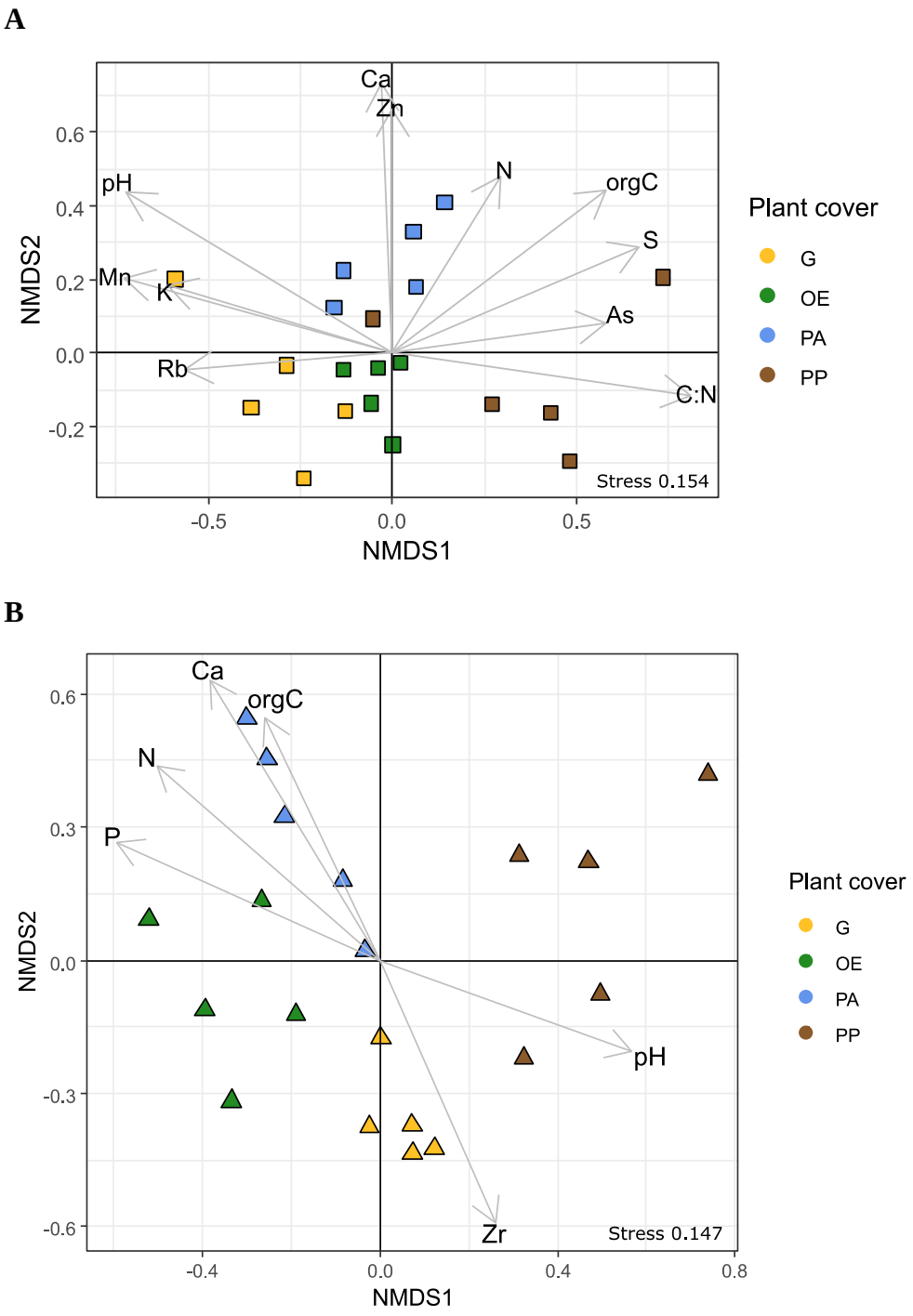


Figure S5.4 Non-metric multidimensional scaling (NMDS) ordination plot of fungal communities according to plant cover factor. A) North site. B) South site. Symbols according to site (N = North (square) and S = South (triangle)) and plant cover (G = Grassland, OE = *Olea europaea*, PA = *Populus alba*, PP = *Pinus pinea*). Significant (p < 0.05) soil properties are presented as arrows.

Table S5.4 Summary of fungal Operational Taxonomic Units (OTUs) and Reads classified according to their functional guilds from FunGUILD database.

	OTUs		Reads	
Guilds	Number	Percentage	Number	Percentage
Saprotroph	874	68.12	729522	37.45
Plant pathogen	175	13.64	136101	6.99
Ectomycorrhizal	119	9.28	1038103	53.29
Animal Pathogen	34	2.65	14626	0.75
Fungal Parasite	18	1.40	1455	0.07
Arbuscular Mycorrhizal	17	1.33	2993	0.15
Lichenized	17	1.33	3205	0.16
Endophyte	12	0.94	9345	0.48
Orchid Mycorrhizal	10	0.78	11840	0.61
Ericoid Mycorrhizal	4	0.31	645	0.03
Epiphyte	2	0.16	25	0.00
Lichen Parasite	1	0.08	61	0.00
TOTAL GUILDS	1283	100	1947921	100
(Undefined)	7125			
(Possible)	616			
(Multiple guilds)	404			
TOTAL	9428			

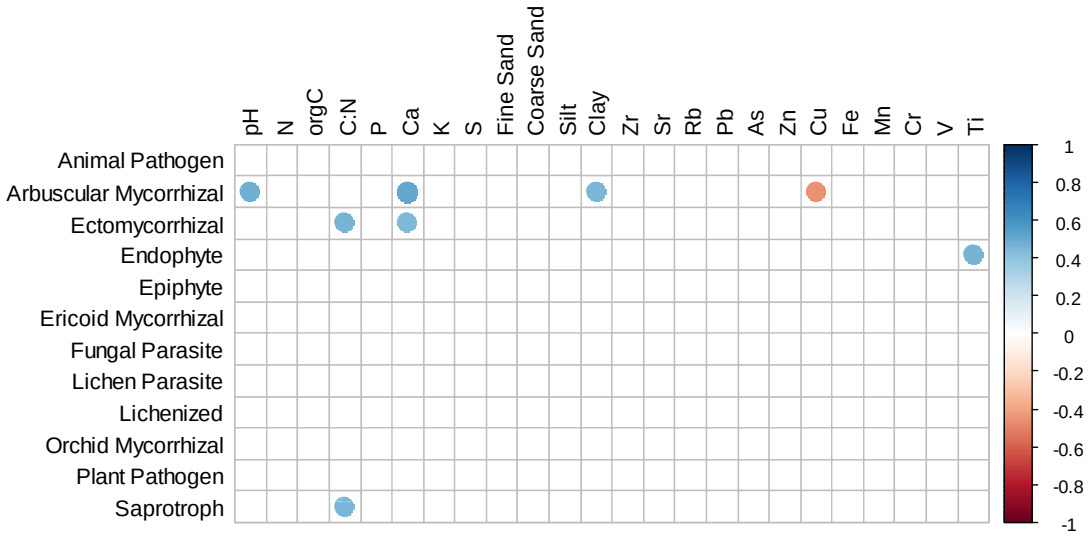


Figure S5.5 Pearson´s correlation coefficient between functional guilds abundance and soil abiotic variables excluding bare soil (n = 40).

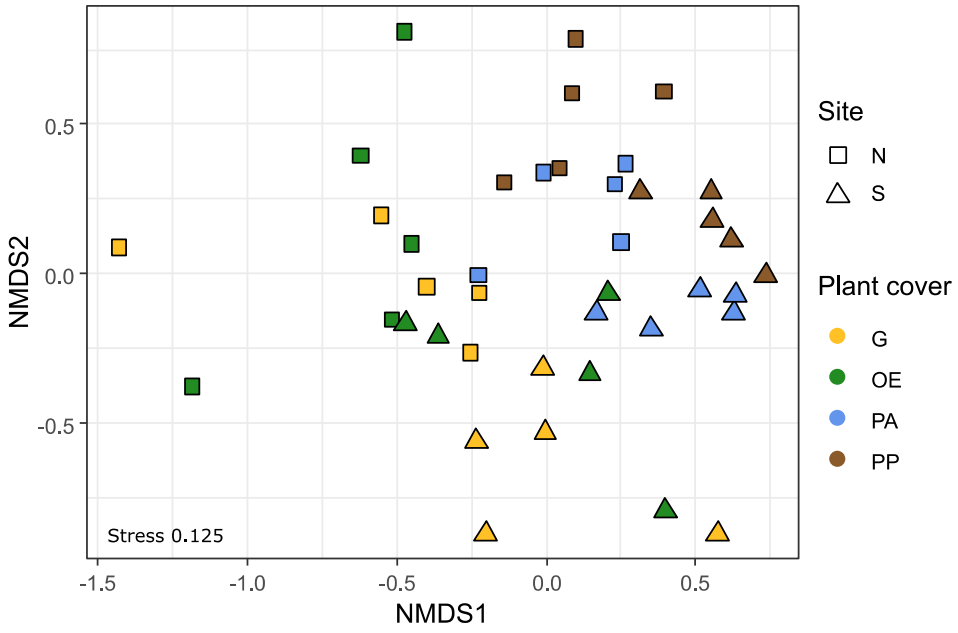


Figure S5.6 Non-metric multidimensional scaling (NMDS) ordination plot of ectomycorrhizal fungal communities according to plant cover and site factors. Symbols according to site (N = North (square) and S = South (triangle)) and plant cover (G = Grassland, OE = *Olea europaea*, PA = *Populus alba*, PP = *Pinus pinea*).

Table S5.5 Two-way analysis of variance (ANOVA) statistic F with effects of Site (North and South) (*df* = 1), Plant cover (Grassland, *Olea europaea*, *Populus alba* and *Pinus pinea*) (*df* = 3) and their interaction (S x Pc) (*df* = 3) on soil abiotic (including trace elements) and biotic variables (n = 40). Tukey's honest significant difference (HSD) *post hoc* with letters indicating differences of Plant cover factor. Asterisks indicate the significance level (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$). δ: Non-parametric Schneirer-Ray-Hare test and H statistic; ¥: Box-cox transformation; α: *VarIdent* function for homocedasticity. orgC: organic carbon; DH: Dehydrogenase activity; BR: Basal Respiration.

Soil variables	Site	Plant cover					F			
		Bare soil (Control)	Grassland	*Olea europaea*	*Populus alba*	*Pinus pinea*	Plant cover	Site	Interaction	
Abiotic										
pH	North	*3.22±0.11*	5.68±0.16	5.59±0.16	5.71±0.20	4.43±0.49	0.26	**296*** **	2.19	δ
	South	*2.55±0.11*	6.93±0.04	6.90±0.05	6.89±0.04	6.98±0.06				
N	North	*0.070±0.012*	0.198±0.041**B**	0.233±0.028**AB**	0.301±0.030**A**	0.233±0.030**AB**	**3.80***	1.00	0.35	
(%)	South	*0.137±0.023*	0.180±0.019	0.246±0.022	0.265±0.037	0.192±0.021				
orgC	North	*0.67±0.09*	2.19±0.41**B**	3.02±0.37**AB**	3.89±0.37**A**	3.76±0.29**A**	**6.00****	0.12	0.28	
(%)	South	*0.79±0.13*	2.53±0.32	3.34±0.35	3.89±0.45	3.48±0.48				
C:N	North	*9.92±1.05*	11.37±0.31**B**	12.97±0.29**AB**	12.93±0.26**A**	16.68±1.13**A**	**16.5*****	**4.06***	2.62	δ
	South	*5.85±0.27*	14.63±2.44	13.53±0.75	14.86±0.47	17.98±1.38				
P	North	*6.04±1.12*	5.32±0.85	6.56±0.85	10.36±1.38	10.16±2.58	3.18	**20.1*****	3.05	
(mg kg^{-1})	South	*2.00±0.51*	13.16±2.36	24.62±4.70	18.78±2.79	14.6±1.45				
Ca	North	*12774±813*	12187±481**B**	12096±435**AB**	16328±1068**A**	12637±1821**B**	**6.25****	**1000*****	1.60	¥
(mg kg^{-1})	South	*19024±1225*	52912±3238	60111±2681	62866±2460	53071±3147				
K	North	*13487±329*	15101±645	14254±450	13927±568	13818±705	1.00	3.82	0.83	
(mg kg^{-1})	South	*12673±100*	15018±456	15274±709	15597±336	14383±613				
Zr	North	*223±9*	250±24**A**	223±11**AB**	202±24**B**	226±16**AB**	**3.37***	**4.49***	0.07	
(mg kg^{-1})	South	*254±24*	279±12	239±6	228±14	250±11				
Sr	North	*109±4*	115±2**AB**	118±3**A**	113±3**AB**	105±4**B**	**3.08***	**13.05****	1.61	

(mg kg^{-1})	South	*103±4*	115±3	127±3	123±4	120±5				
Rb	North	*58.19±1.67*	68.56±3.81	67.44±0.50	61.75±2.46	63.93±1.73	1.43	0.60	0.96	
(mg kg^{-1})	South	*64.81±2.63*	65.3±1.84	69.29±1.83	66.15±2.48	66.04±2.65				
Pb	North	*290±59*	167±15	168±15	174±4	188±19	0.30	**85.8*** **	0.83	¥α
(mg kg^{-1})	South	*5715±2514*	92.7±14.9	93.3±12.9	80.2±13.2	75.7±5.3				
As	North	*196±32*	92.8±7.6	105±9	112±26	121±20	0.39	**98.89*** **	0.89	¥α
(mg kg^{-1})	South	*957±141*	44.0±7.6	44.7±6.4	39.9±6.4	37.6±3.3				
Zn	North	*153±15*	255±20	250±6	310±27	249±33	1.15	0.67	0.38	
(mg kg^{-1})	South	*187±46*	251±31	252±16	263±33	239±23				
Cu	North	*124±8*	144±10	159±8	143±11	135±7	0.95	**275*** **	0.78	
(mg kg^{-1})	South	*98.1±13.5*	54.5±8.7	53.3±2.8	50.2±7.4	52.3±5.6				
Fe	North	*47110±3776*	41941±2164	42006±1939	39664±3382	39763±1395	0.34	**159*** **	0.30	¥
(mg kg^{-1})	South	*96279±9864*	21812±1756	23971±1475	22430±1708	23220±1611				
Mn	North	*366±22*	672±56	599±55	567±60	486±55	1.45	**61.9*** **	1.53	
(mg kg^{-1})	South	*127±10*	331±32	335±27	322±33	336±24				
Cr	North	*41.00±11.07*	46.59±8.04	30.92±4.57	44.77±10.11	43.86±2.85	0.82	**7.46***	1.06	
(mg kg^{-1})	South	*30.92±1.51*	25.12±3.23	30±4.78	32.85±3.34	32.43±2.83				
V	North	*125±7*	135±5	124±6	124±8	110±6	1.51	**47.51*** **	1.43	
(mg kg^{-1})	South	*82.6±6.0*	88.8±6.8	90.7±5.5	98.3±5.3	89.3±7.8				
Ti	North	*4873±194*	5128±396	4957±334	5110±390	4270±347	1.12	**47.63*** **	0.98	
(mg kg^{-1})	South	*3251±131*	3569±177	3308±185	3471±137	3460±218				
S	North	*12332±2097*	1795±330	2147±402	4324±1715	4350±1301	0.36	**52.9*** **	2.01	¥α
(mg kg^{-1})	South	*47928±2811*	1000±168	880±129	994±143	831±76				
Biotic										
DH	North	*0.87±0.13*	3.95±0.57	5.03±0.29	5.36±0.66	4.01±0.66	2.87	**12.4** **	0.36	
(μg INTF g^{-1}h^{-1})	South	*0.20±0.05*	2.90±0.14	3.49±0.43	3.96±0.37	3.35±0.34				
BR	North	*NA*	2.05±0.74	2.8±0.60	3.04±1.07	2.19±0.81	1.03	4.99	0.16	

(μg C $g^{-1}h^{-1}$)	South	*NA*	2.92±0.32	3.46±0.20	3.49±0.39	3.47±0.19			
Texture									
Fine sand	North	*24.02±2.82*	21.74±4.63	21.72±5.73	16.78±3.65	18.32±5.11	0.60	**5.41***	0.15
(%)	South	*21.96±0.94*	27.08±3.04	28.30±3.47	26.20±2.33	22.90±1.98			
Coarse sand	North	*44.40±7.49*	40.52±9.02	47.76±9.87	55.16±8.50	49.96±8.81	0.55	**33.16*****	0.50
(%)	South	*19.14±1.93*	20.20±4.65	13.96±4.30	19.32±1.58	23.82±5.94			
Silt	North	*20.16±3.02*	27.72±3.31	24.36±3.93	20.04±4.02	22.04±2.64	1.02	**26.00*****	0.69
(%)	South	*46.00±4.51*	33.28±2.20	36.18±1.62	33.26±1.46	32.28±2.24			
Clay	North	*11.44±2.01*	9.98±1.72	6.10±0.78	7.96±2.32	9.64±2.07	0.26	**105.18*****	1.13
(%)	South	*12.84±1.98*	19.42±1.57	21.50±0.62	21.20±1.81	20.96±1.97			

6. DIVERSIDAD FUNCIONAL DE HONGOS ECTOMICORRÍCICOS

Resumen

Los estudios basados en rasgos son herramientas muy útiles para explicar las reglas ecológicas de ensamblaje y el funcionamiento del ecosistema. Sin embargo, su uso en la microbiota del suelo no ha sido todavía explorado en profundidad. En este estudio exploramos los cambios funcionales basados en los rasgos de las comunidades de hongos ectomicorrícicos en simbiosis con la encina (*Quercus ilex* subsp. *ballota*) en un suelo contaminado por elementos traza.

La variación de la composición de las especies ectomicorrícicas estuvo determinada por el carbono, el calcio y la contaminación del suelo; sin embargo, la diversidad taxonómica no dependió del nivel de contaminación. Los valores de los rasgos medios de las comunidades ectomicorrícicas mostraron una disminución en la formación de rizomorfos e hifas al aumentar la contaminación, y la comunidad convergió hacia especies con un desarrollo de rizomorfos menos frecuente. Sugerimos que la contaminación por elementos traza pudo actuar como el principal filtro ambiental en la diversidad de los rasgos de la comunidades de hongos ectomicorrícicos. Los efectos de las propiedades del suelo, como el carbono, también produjeron un efecto en los valores medios de las hifas pero no causó convergencia en su distribución.

En resumen, encontramos una reducción de la diversidad funcional en las comunidades de hongos ectomicorrícicos debido a la contaminación con un efecto potencial en el funcionamiento del ecosistema. Estos descubrimientos apoyan el potencial de los estudios basados en rasgos para evaluar los cambios en la diversidad funcional de las comunidades microbianas.

Abstract

Trait-based approaches are useful tools to explain ecological assembly rules and ecosystem functioning. However, their use for soil microbiota has not been explored in depth yet. We explored trait-based functional changes of ectomycorrhizal (ECM) fungal communities associated with holm oak (*Quercus ilex* subsp. *ballota*) in a trace element contaminated area.

We found a variation in ECM fungal species composition determined by soil C, Ca and trace elements; however, taxonomic diversity was not dependant on contamination level. Mean trait values of ECM fungal communities showed less rhizomorph and emanating hyphae production when increasing contamination, and the community converged towards species developing rhizomorphs less frequently. We suggest that trace elements in soils acted as the main environmental filter of trait diversity of ECM fungal communities. The effect of soil nutrients, i.e. soil C, affected the community mean trait values of emanating hyphae but did not cause a convergence in its distribution.

In summary, we found a reduction in the functional diversity of ECM fungal communities due to trace element contamination with potential to affect ecosystem functioning. This finding supports the potential of trait-based approaches to assess changes in the functional diversity of soil microbial communities.

6.1. Introduction

Trait-based approaches are excellent tools to disentangle community assembly rules and to link community composition, environmental changes and ecosystem functioning (Díaz & Cabido, 2001; Garnier et al., 2016; Lavorel et al., 2013). The basic principle of trait-based approaches relies on the use of functional traits of organisms, instead of mere species abundance counts, to describe emergent properties of ecosystems (Cadotte et al., 2011). Environmental constraints are known to affect the taxonomic diversity of communities by filtering the species according to their traits -i.e. response traits-, promoting the convergence of species with similar traits, in a process known as environmental filtering (Götzenberg et al., 2012). On the other hand, functional traits that have the potential to change ecosystem functioning are considered effect traits. The degree to which response and effect traits are interrelated determines the possible consequences of environmental filtering (Lavorel and Garnier, 2002).

In plant ecology, the links between plant traits and ecosystem functioning have been widely explored during recent decades (Díaz et al., 2007). Most studies have been focused on aboveground traits (Bardgett et al., 2014; Laliberté, 2016) and only more recently the "hidden" belowground plant functional diversity has started to be addressed (e.g. Bu et al., 2016; de la Riva et al., 2017; Gould et al., 2016). Indeed, the few studies addressing the belowground compartment of plant communities has, ranging from the level of organisms to that of ecosystems, highlighted the methodological potential for explaining ecosystem functioning (e.g. López-García et al., 2014; Mulder et al., 2005; Pelosi et al., 2014; Santorufo et al., 2015). Despite the growing interest, trait-based studies of soil organisms face important challenges especially due to the difficulties associated to the direct trait measurements of individual organisms, especially in the case of microbes (see Crowther et al., 2014).

Ectomycorrhizal (ECM) fungi are important components of terrestrial ecosystems: they are symbiotic nutrient suppliers of trees dominating in wide areas of the globe (van der Heijden et al., 2015). Their impact in ecosystems is not only limited to nutrient (mainly N) and water uptake from the soil, but they also participate in aspects of C cycling such as C sequestration (Clemmensen et al., 2013) and organic matter degradation (Tunlid et al., 2017). It has been suggested that their implications for ecosystem processes can be

mediated by specific fungal traits which, in turn, are affected by environmental changes (Koide et al., 2014). In particular, the way in which ECM fungal species invest in morphological structures determines the hyphal exploratory capacity. Agerer (2001; 2006) distinguished four broad categories of exploration types: contact, short, medium and long distance, as a function of the morphology and development of emanating hyphae and rhizomorphs, i.e. specialised hyphal cords for long distance transport of water and nutrients, in the soil. The relative abundance of species with different exploration types is determined by the nutrient status of soils (Hobbie and Agerer, 2010; Moeller et al., 2014; Suz et al., 2014). Indeed, fungi exhibiting different exploration types usually harbour different enzyme activities (Tedersoo et al., 2012). Additionally, it has been suggested that ECM exploration type drives long term C sequestration due to differences in biomass production and turnover among them (Clemmensen et al., 2015; Koide et al., 2014). Another relevant trait with implications for ecosystem processes is the melanin content in cell walls, which is considered a protective trait against multiple abiotic stressors (Treseder and Lennon, 2015) such as enzymatic degradation (Rosas and Casadevall, 2000), salinity (Kogej et al., 2006), water stress (Fernandez and Koide, 2013) and even ionising radiation (Cordero, 2017). Melanin content is inversely related to the decomposition rates of fungal necromass due to its recalcitrant nature (Fernandez and Koide, 2014), and thus it has the potential to influence C storage in soil, acting as an effect trait (Clemmensen et al., 2015). The morphological structure of ECM allows the characterisation of individual root tips that consists of single fungal species. Previous studies have attributed categorical trait information, usually extracted from databases, to each ECM fungal taxa (Aguilar-Trigueros et al., 2014; Kjøller et al., 2012) thereby ignoring the intraspecific variation and plasticity of these traits. As far as we know, only one recent study (Courty et al., 2016) has used direct trait characterisation of individual ECM root tips to develop a trait-based analysis. In that work, the authors demonstrated that extracellular enzyme traits at ECM fungal community level can be driven by the soil nutrient status.

Studies on ECM functional diversity have mainly focused on the impact of soil nutrient status and the natural succession of ECM fungal communities (Clemmensen et al., 2015; Kjøller et al., 2012; Moeller et al., 2014; Suz et al., 2014). However, the effect of trace elements, mainly heavy metals, on ECM fungal community composition and diversity has been scarcely studied and the results are controversial. Hui et al. (2011) and Op De Beeck et al. (2015) did not find any effect of heavy metal contamination on ECM

taxonomic diversity but noted a shift in the species composition of their communities. In contrast, Sousa et al. (2014) found both, an effect on community composition and an increase in ECM fungal diversity in Cd-contaminated plots. However, Huang et al. (2012) did not find a clear effect of the contamination neither on community composition nor at the taxonomic richness level. Despite some influences on taxonomic diversity, there exists a gap of knowledge on how such kind of anthropogenic impact affects the functional diversity of ECM fungal communities. Trace elements are likely to filter against the ECM fungal species spreading more intensively in soils (those producing emanating hyphae and/or rhizomorphs) due to an increased exposure to trace element toxicity (Pawlowska and Charvat, 2004). In addition, increased melanisation of ECM fungal communities would be expected as a consequence of the known protective effect of melanin against heavy metals (Gadd and Rome, 1988; Galli et al., 1994).

Here we determined hyphal exploration types and melanisation level as traits of ECM fungal species, molecularly identified, associated with holm oak (*Quercus ilex* subsp. *ballota*) in a restored trace element contaminated site (Guadiamar River valley, South of Spain). We quantified exploration type by microscopically confirming the presence of emanating hyphae and rhizomorphs on single ECM root tips. Our hypotheses were that: i) higher concentrations of trace element in soil reduce the taxonomic diversity of ECM fungal species and shifts the community composition; ii) there is an effect of trace element contamination on the community mean traits towards shorter exploration types and more melanised fungi; iii) we expect that trace element contamination reduces the trait dispersion in ECM fungal communities, since it acts as a filter of species according to their traits.

6. 2. Material and Methods

6.2.1. Study area

The Guadiamar Green Corridor was remediated and afforested with autochthonous woody plant species (Domínguez et al., 2008). Only two patches unaffected by the mine spill were included in the reforestation program and planted with identical vegetation, one

in the north of the dam breakdown, to allow connection of the corridor with other natural areas, and one in the south of the corridor, where an entire piece of land was expropriated including contaminated and non-contaminated surface.

The affected area had two contrasting geologically-based sites, the North site and the south site, that were remediated following the same criteria. See the general description of the study site in chapter 3.

The vegetation in the Corridor is dominated by sclerophyllous Mediterranean forests with the ectomycorrhizal holm oak (*Quercus ilex* subsp. *ballota*) as the most representative species. The area covered by the toxic flood was agricultural, however patches of agro-forest (*Quercus ilex*) and natural Mediterranean vegetation were closely distributed along the corridor ranging from hundreds of meters to one km maximum distance.

6.2.2. Sample design, collection and processing

Four different areas were sampled: two acidic in the South site, one affected by the mine spill and the other unaffected, and two calcareous in the South site, also affected and unaffected by the mine spill (Fig. S6.1). The choice of these four sampling sites made possible to construct a gradient of contamination availability due to different exposure to contamination and the variability across sites (dependent on the original soil nature - slightly acidic vs. calcic-), that makes harmful effects of contamination vary (as shown by Domínguez et al. 2017). The selection of sites were also hampered by the low availability of sites in which enough trees got established and had a similar spatial distribution (tree mortality rates were high the first two years after plantation, see Domínguez et al., 2010).

Our sampling was focused in sampling and characterizing individual holm oaks due to its constant presence all along the corridor and its representativeness of this dry Mediterranean region. All trees had been planted at the same time and from similar seed provenance. Keeping the host species constant, we could focus on the soil variability across the studied area, thus excluding other confounding factors such as plant host identity and age (Albornoz et al., 2016; Davey et al., 2015). Ten trees were randomly selected in each site (Fig. S6.1 and Table S6.1). In April 2016, roots of trees were sampled by carefully tracing them from the stem of the tree in the four cardinal directions and ca.

200 g root material was collected from each direction, i.e. subsamples. Soil samples (0-20 cm depth) were taken with an auger from the four directions under each tree canopy projection, and were pooled to a total of 500 g to make a composite sample per tree.

6.2.3. Soil analyses

All soil samples were air-dried and sieved to <2 mm for physico-chemical analysis. Soil pH was measured in a 1:2.5 soil-water suspension after shaking for 30 min. Total C and N content was determined using a Flash HT Plus elemental analyser. Carbonate content was measured by the manometric method (Demolon and Leroux, 1952); soil organic C was then calculated as the difference between total C and the C contained in carbonates. Ammonium and nitrate were extracted by 1M KCl and determined by multiparametric Bran-Luebbe autoanalyser (Maynard et al., 2007). Olsen method (Olsen et al., 1954) was used for available P estimation in neutral and basic soils and Bray method was used in acidic soils (Bray et al., 1945). Available K, Ca and Mg were extracted with 1 M ammonium acetate and determined by atomic absorption spectrophotometry. Sulphur and pseudo-total trace element concentrations in soil samples (ground to <60 μm) were determined by digestion with aqua regia (1:3 v/v conc. HNO_3/HCl) in a Digiprep MS block digestor (SPS Science) equipped with a temperature-time programmable controller and polypropylene digestion tubes. Trace elements in extracts were determined by ICP-OES.

6.2.4. Mycorrhizal determinations

The seven longest root fragments in each of the four subsamples were selected to make a composite sample of 28 fragments per tree. The extreme left mycorrhizal root tip of each root fragment was photographed for further trait quantification (Supplementary material Methods) and a small portion of each individual root tip was cut and immersed separately into 10 µl of Extraction Solution (Extract-N-Amp™ Plant PCR Kit by Sigma-Aldrich) for subsequent molecular identification. Photographs of individual root tips were used to record the presence/absence of emanating hyphae and rhizomorphs in each root tip. The

colour of root tips was assessed in the CMYB scale using ColorPick v. 3.0 (http://www.iconico.com/colorpic/; see detailed description of methodology at Supplementary material Methods) and the black colour content annotated for each root tip (ranging from 0 to 1). The darkness of the root tips, or the content in black colour, is directly related with the melanin content of fungi in accordance with classical visual criteria used to differentiate between melanised and non-melanised fungi (e.g. Fernandez et al., 2016). When applying our colorimetric approach to the photographs published by Fernandez and Koide (2014), we found a high correlation between black colour and the melanin contents quantified in that publication (Supplementary material Methods).

6.2.5. Molecular analyses

Tubes containing individual root tips and Extraction Solution were subjected to a heat shock (95 ºC for 10 min, 20 ºC for 10 min) followed by the addition of 10 µl of Dilution Solution (Extract-N-Amp™ Plant PCR Kit by Sigma-Aldrich) and frozen until PCR setup. PCR amplification was carried out using 0.55 µl of DNA template with a Illustra PureTaq Ready-To-Go bead (GE Healthcare UK Limited, Buckinghamshire, UK) and 0.8 µM of primers ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990) in a final volume of 25 µl. The thermocycling program was as follows: 3 min initial denaturation at 94°C; 35 cycles of 30 s denaturation at 94°C, 35 s annealing at 53°C and 1 min elongation (increased in 5 s each cycle) at 72°C; and a 4 min final elongation (as described by Suz et al., 2014). PCR products were purified using MEGAquick-spin (Intron Biotechnology, South Korea) and Sanger sequenced in the Unidad de Genómica y Síntesis de DNA, Instituto de Biomedicina y Parasitología López Neyra, CSIC (Granada, Spain). Sequence chromatograms were checked individually and those presenting double peaks, i.e. containing more than one fungal sequence, were discarded. In these cases, a new root tip was picked up randomly from the root sample to ensure a minimum number of sequences per root sample. The remaining sequences were blasted against the UNITE database (Koljalg et al., 2005) and those found corresponding to ECM fungi were grouped by genera or family. Sequences in each taxonomical group were aligned separately using MAFFT v. 7 (Katoh and Standley, 2013) and clustered in MOTHUR v. 1.35.1 (Schloss et al., 2009) at a 97% cut-off to delimitated Operational Taxonomic Units (OTU). DNA sequences were compared against the UNITE database (Koljalg et al., 2005) for their

taxonomic placement and Species Hypothesis determination. ECM fungal sequences were deposited in GenBank (http://www.ncbi.nlm.nih. gov/genbank/) under accession numbers MG273770-MG274263.

6.2.6. Data analyses

The whole analysis was based in the use of continuous data coming from the individual characterization of holm oak trees. For a broad characterization of study plots, a principal components analysis was carried out after log-transforming of trace element and soil variables. Differences in abiotic and biotic (i.e. ECM fungal traits) variables across plots were assessed by ANOVA after checking for normality and homoscedasticity. Tukey´s Honest was used as *post hoc* test. Non-normal variables were log or square root transformed. Variables that even when transformed were not normally distributed were analysed by non-parametric Kruskal Wallis test with pairwise Dunn test corrected using Bonferroni as *post hoc*.

The OTU abundance data matrix was constructed based on the number of root tips where each species was identified. A rarefaction analysis was carried out to ensure a high and even coverage of the total diversity of OTUs in each plot. The abundance matrix was Hellinger transformed for subsequent analyses (Legendre and Gallagher, 2001). Species richness (S), Chao1 and Simpson (1-D) indices were calculated as alpha diversity measures.

An OTU × trait matrix was constructed by calculating the frequency of emanating hyphae and rhizomorphs in the total root tips of each ECM fungal OTU. The black colour percentage was used as a proxy of melanin content and its value for each species was calculated as the average of the black component across all identified root tips per each OTU. To scale up from OTU to community level, all these traits were weighted by the relative abundance of each OTU to calculate community-weighted means (CWMs) of mycorrhizal traits for each tree (called fixed trait averages by Lepš et al., 2011).

A Variation Partitioning approach (Legendre and Legendre, 1998) was used to assess the influence of soil variables and trace elements on species (species-based RDA) and trait distribution (CWM-based RDA) (Kleyer et al., 2012). For that, every abiotic variable was log transformed, with the exception of pH, and the Hellinger transformed OTU matrix

and the CWM matrix were used as response matrices for the species- and CWM-based RDAs, respectively. A previous selection of variables was carried out by stepwise model building for constrained ordination methods (Blanchet et al., 2008) with backward and forward selection to include important variables only. Since the objective of this analysis was to quantify the relative contribution to OTU and CWM distribution of soil background variables, trace elements and their shared covariation, the approach was applied separately for each group of soil factors (soil background variables and trace elements). For each subset of variables selected by the models, the variance inflation factors (VIF) were calculated (Gross, 2003), and variables above VIF = 5 were removed. To control for the effect of spatial distribution of samples, principle coordinates of neighbour matrices (PCNM approach; Borcard and Legendre 2002) were calculated. The resulting PCNM axes were subjected to the same selection as described for soil and trace element variables, and those found to significantly influence the OTU or CWM distribution were selected. Every selected variable, either from soil, trace elements or spatial components, were feed to the variation partitioning analysis. To visualise the identified trends, an RDA ordination was carried out including all selected variables.

To assess the significance of each of the soil background variables and trace elements on fungal trait values, RLQ and fourth-corner analyses were performed (Legendre et al., 1997; Dray and Legendre, 2008). This method directly compares the three matrices: environmental, species abundance and species traits. Effects were calculated using permutation model #6 with 9999 permutations, which is a combination of models #2 (permutes values of sites) and #4 (permutes values of species) which does not have an inflated type I error (Dray and Legendre, 2008; ter Braak et al., 2012). False discovery rate correction for multiple testing (Benjamini and Yekuteli, 2001) was applied.

In order to obtain insights into the rules governing ECM fungal community assembly, the trait distribution across OTUs in communities was compared with random expectations. For that, standardised effect size of mean pairwise distance (ses.mpd) between OTUs in each community was calculated by using the OTU abundance data matrix and a Euclidean trait distance matrix between OTUs. *Independent swap* algorithm was used to generate null communities (Gotelli, 2000). Ses.mpd varies from -1 to 1, where negative values mean trait convergence and positive values trait divergence. Relationships of ses.mpd

with soil factors were checked by Pearson correlation applying a false discovery rate correction for multiple testing (Benjamini & Yekuteli, 2001).

All statistics were carried out in R software v 3.3.2 (R Development Core Team) using *vegan* (Oksanen et al., 2012), *picante* (Kembel et al., 2010) and *ade4* (Dray and Dufour, 2007) packages.

6.3. Results

6.3.1. Soil abiotic factors

The two sites affected by the mine spill (CN and CS) showed significantly higher values of most of the measured pseudo-total trace element concentrations (As, Cd, Cu, Pb, S and Zn) in relation to the non-affected sites (UN and US) (Table 6.1, Fig. 6.1). However, when looking at other soil variables, the sites from the North site (CN and UN) had relatively similar values of pH, NH_4 and total N - more acidic and N-rich -, than those from the South site, CS and US (Table 6.1, Fig. 6.1).

Table 6.1 Mean values of soil variables (±SE) in the two studied plots affected and the two unaffected by the toxic mine spill of Guadiamar river (SW Spain). Contaminated north (CN) and south (CS), uncontaminated north (UN) and south (US). ANOVA analysis is displayed in last two columns (F and *P*). Means not sharing a letter in common differ significantly according to the Tukey´s Honest *post hoc*.

	Contaminated plots		**Uncontaminated plots**		**ANOVA**	
Soil variables	**CN**	**CS**	**UN**	**US**	**F**	**P values**
pH	4.84±0.23c	6.97±0.15a	6.26±0.13b	7.33±0.03a	51.48	<0.001
Ca (mg kg^{-1})	1,890±270b	4,890±90a	2,190±520b	3,240±410b	13.39	<0.001
K (mg kg^{-1})	139.16±18.33b	212.01±12.71ab	286.11±27.93a	235.92±18.39a	9.25	<0.001
Mg (mg kg^{-1})	97.02±8.27c	193.21±5.76b	203.99±29.82b	289.54±29.20a	15.68	<0.001
P (mg kg^{-1})	12.72±1.28	8.12±0.88	10.38±1.71	17.17±4.75	0.72	0.547
$CaCO_3$ (%)	0.55±0.06c	8.13±0.38a	1.20±0.13b	1.41±0.24b	133.1	<0.001
NH_4(mg kg^{-1})	4.77±0.34a	2.87±0.17b	5.07±0.57a	3.49±0.28b	10.55	<0.001
NO_3 (mg kg^{-1})	4.78±1.35a	2.49±0.45a	2.64±0.47a	1.21±0.19b	4.27	0.011
Total C (%)	1.72±0.16b	2.04±0.08a	1.56±0.13b	1.02±0.09c	11.93	<0.001
Total N (%)	0.16±0.02a	0.11±0.00b	0.15±0.01a	0.10±0.01b	10.65	<0.001
Total trace elements						
As (mg kg^{-1})	161.83±21.71a	40.39±4.98b	18.03±1.27c	13.52±1.09c	97.26	<0.001
Cd (mg kg^{-1})	0.68±0.11a	0.67±0.07a	0.21±0.03b	0.02±0.01c	43.62	<0.001
Cu (mg kg^{-1})	192.55±7.82a	58.15±5.70b	40.54±4.46b	18.69±1.72c	211	<0.001
Fe (mg g^{-1})	40.48±2.14a	21.97±0.57c	27.52±0.93ab	22.80±1.50bc	27.57	<0.001
Mn (mg kg^{-1})	391.53±39.47b	414.78±15.41b	851.88±29.62a	486.40±47.77b	37.09	<0.001
Ni (mg kg^{-1})	13.01±0.70b	14.60±0.44b	21.69±1.04a	15.73±1.23b	17.44	<0.001
Pb (mg kg^{-1})	274.40±37.54a	76.66±8.26b	57.57±6.87b	19.82±1.14c	89.89	<0.001
S (mg g^{-1})	3.12±0.41a	0.71±0.09b	0.17±0.02c	0.10±0.01c	123.3	<0.001
Zn (mg kg^{-1})	228.99±29.61a	229.65±21.54a	96.93±9.25b	44.43±3.71c	66.39	<0.001

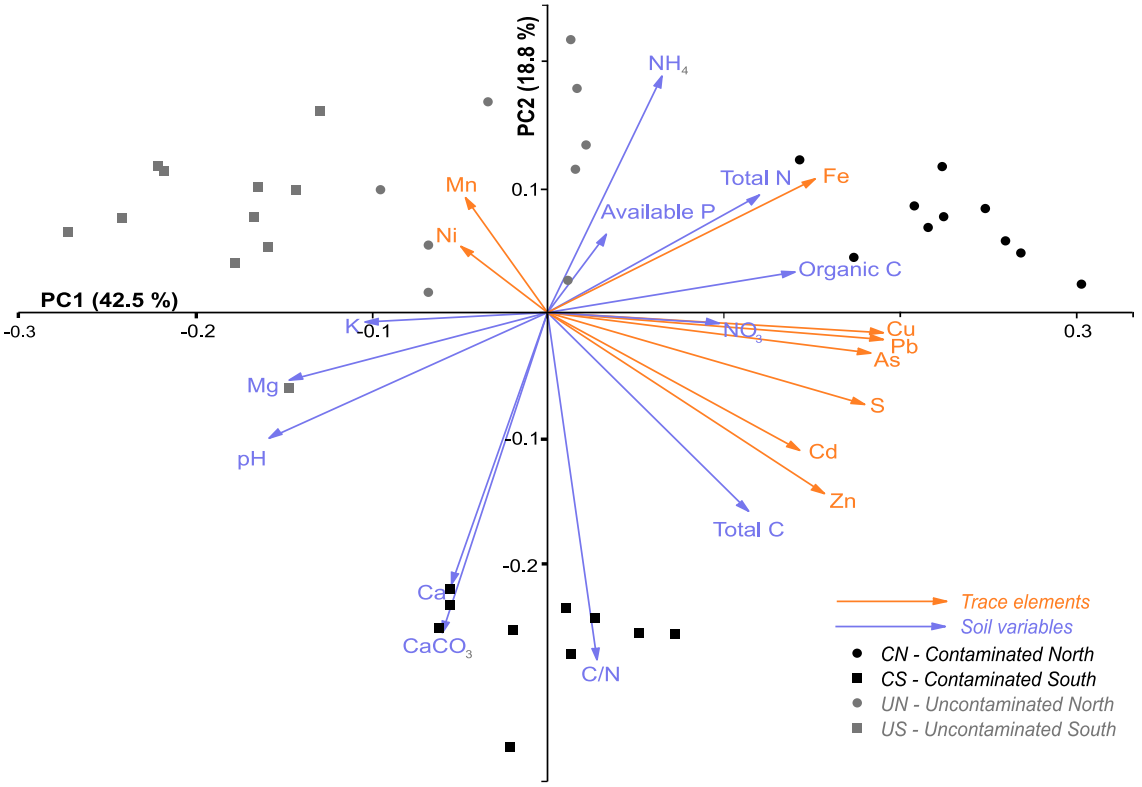


Figure 6.1 Principal component analysis (PCA) of soil variables and trace elements in four locations across the Guadiamar river valley (SW Spain) which differ in exposure to contamination by trace elements and inherent soil background variables.

6.3.2. ECM fungal community composition, taxonomic and functional diversity

From a total of 1,120 sampled root tips, 494 produced successful PCR amplifications and were identified as ECM fungal species. They were classified into 55 different OTUs belonging to 14 families and 19 genera (Table S6.2). There were two species which dominated the communities: *Hebeloma cavipes* and *Thelephora terrestris*, representing 16.4% and 12.3% of sequences, respectively. Most of the species occurred on less than two trees (Table S6.2). Rarefaction analysis showed that for each site, most of the OTU richness was recorded (Fig. S6.2). The mean number of ECM fungal species per tree was 3.8, the estimated Chao richness was 4.9 species per tree, and the Simpson dominance index averaged 0.6. For the three diversity measures there were no significant differences between sites or contamination levels.

The frequencies of emanating hyphae and rhizomorphs across OTUs ranged from 0 to 100 %, and melanisation from 64 to 94.7 % (Table S6.3). Among the three most abundant families (Cortinariaceae, Russulaceae and Thelephoraceae), OTUs in the Cortinariaceae family showed the lower variability in the three studied traits (emanating hyphae (%): 66.6 to 100; rhizomorphs (%): 0 to 66.6; melanisation (%): 64.6 to 72.9). OTUs belonging to the other two dominant families were highly variable in terms of emanating hyphae and rhizomorphs (ranging 0 % to 100 %), while melanisation spanned in the range between 70 % and 94.7 %. The two most dominant species (*H. cavipes* and *T. terrestris*) had similar rhizomorph frequency and melanisation (around 12 % and 68 %, respectively), but *H. cavipes* showed emanating hyphae more frequently (95.1 %) than *T. terrestris* (88.5 %). The trait ranges exhibited by the detected ECM fungal species were congruent with the available descriptions of species and genera (Deemy database, see Table S6.3 for a comparison).

6.3.3. Effect of abiotic variables on ECM fungal community composition

According to the selected RDA models, the variables that best explained ECM fungal community variability (OTU matrix) were available Ca, organic C and total C among soil background variables, and Cu, Ni, S and Zn among trace elements (Fig. 6.2a; Table S6.4). Sulphur was removed from the subsequent analysis due to a high VIF result. Two PCNM axes were found to influence OTU community composition. The variation partitioning approach revealed that soil background variables and their covariation with trace element explained 8.36 and 0.55 %, respectively; meanwhile trace elements alone explained 3.82 % of variation in the model (Fig. 6.2a, pie chart). The spatial distribution of ECM communities explained a 2.06 % alone, and shared 2.86 % with soil background and trace element variables. There was no sign of collinearity between variables in the variation partitioning analysis. The two most abundant species, *H. cavipes* and *T. terrestris*, showed contrasting patterns regarding the trace element and Ca gradients, respectively, in the RDA ordination (Fig. 6.2a). *H. cavipes* seemed to be related to lower concentrations of Cu, Zn and total C, and higher concentrations of Ni. *T. terrestris* appeared to be related with lower concentrations of Ca, as shown in Fig. 6.2a.

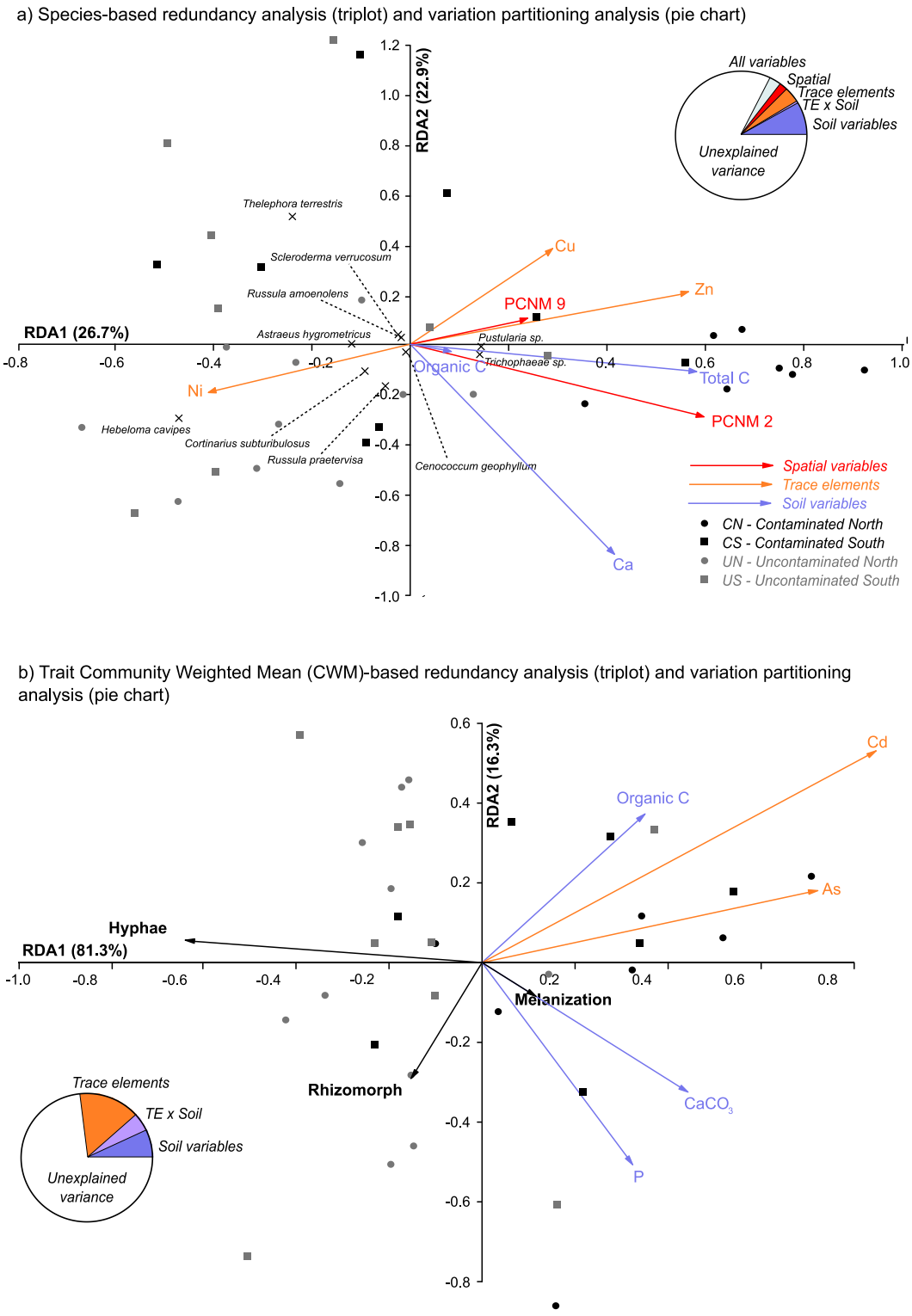


Figure 6.2 Redundancy analysis triplots of ectomycorrhizal (ECM) fungal communities driven by trace element contamination and soil background variables in the Guadiamar river valley (SW Spain). a) Species-based redundancy analysis (triplot) and variation partitioning analysis (pie chart). Species present in less than 5% have not been represented. b) Trait Community Weighted Mean (CWM)-based redundancy analysis (triplot) and variation partitioning analysis (pie chart). The mean frequency of emanating hyphae, rhizomorphs and melanisation (as a function of the black color component) of ECM fungal communities are included in the analysis.

6.3.4. Effect of abiotic variables on ECM fungal community traits

The RLQ analysis showed a significant effect of the abiotic environment on the community composition by an interaction with species traits (model #2, $P = 0.006$; model #4, $P < 0.001$). Significant negative interactions were found between CWM of emanating hyphae and rhizomorphs and some trace elements and total C (displayed in Table 6.2). On the other hand, melanisation significantly interacted with $CaCO_3$.

	Hyphae	**Rhizomorph**	**Melanisation**
As	-0.30*	-0.13	0.10
Cd	-0.33*	-0.27	0.08
Cu	-0.25	-0.17	-0.06
Fe	-0.08	0.00	-0.26
Mn	0.14	0.10	-0.28
Ni	0.14	0.17	-0.27
Pb	-0.28	-0.19	-0.01
S	-0.33*	-0.16	0.09
Zn	-0.35*	-0.16	0.12
pH	0.06	0.12	0.25
$CaCO_3$	-0.19	0.00	0.41*
K	0.05	0.03	0.13
Ca	-0.13	0.12	0.31
Mg	0.08	0.20	0.14
Total C	-0.31*	-0.14	0.14
Organic C	-0.14	-0.12	-0.12
C/N	-0.13	-0.14	0.02
Total N	-0.12	-0.09	-0.18
NH_4	0.07	0.00	-0.18
NO_3	-0.23	-0.25	0.13
P	-0.12	0.16	-0.02

Table 6.2 Results of the fourth corner analysis of the relationships between ectomycorrhizal fungal traits and soil factors in the Guadiamar river valley (SW Spain).

The r values shown indicate the strength of the interactions. Bold letter: $p < 0.10$; *: $p < 0.05$.

The soil background variables that best explained CWM traits distribution included $CaCO_3$, total C, organic C and available P (Fig. 6.2b; Table S6.4), however, total C was removed from subsequent analysis due to a high VIF. On the other hand, among the trace elements, As, Cd and Cu best explained the variation of fungal community traits. Cu was finally removed due to a high VIF. No spatial variables (PCNM axes) were found to significantly explain any variation in trait distribution and were not included in the variation partition analysis. When partitioning the variation into trace element and soil background variables, trace elements explained 15.46% of the total variation, soil background 7.54% and their covariation 6.59% of the trait variability (Fig. 6.2b, pie chart). In agreement with the fourth corner analysis (Table 6.2), emanating hyphae and rhizomorphs appeared negatively related to trace element concentrations and organic C. Meanwhile, melanisation and $CaCO_3$ showed a clear positive covariation (Fig. 6.2b).

The analysis of trait distribution (ses.mpd values) across sites showed no differences among them. The correlation of ses.mpd values of fungal traits with the selected variables in the RDA models (As, Cd, $CaCO_3$, organic C and available P) showed that rhizomorph ses.mpd negatively correlates with Cd concentration (Table 6.3), which means that the ECM species in communities became more similar with increasing Cd concentration. No other significant correlations were found, however emanating hyphae ses.mpd showed a similar magnitude in its positive correlation coefficient with Cd (Table 6.3).

ses.mpd values	Emanating hyphae	Rhizomorph	Melanisation
As	0.183	-0.250	-0.420
Cd	0.427	-0.482*	-0.331
$CaCO_3$	0.104	0.111	0.095
Organic C	0.181	-0.222	-0.396
Available P	0.349	0.360	0.126

Table 6.3 Pearson correlation coefficients between trait distribution of ectomycorrhizal fungal communities, as standardized effect size of mean pairwise distances of communities for each fungal trait, and trace element concentrations and soil variables in the Guadiamar river valley (SW Spain). Only the selected soil variables in the best trait-based RDA model were included. *: $p < 0.05$.

6.4. Discussion

Overall, our trait-based approach proved to be a highly useful tool to quantify potential effects of an environmental disturbance on the functional diversity of natural microbial communities. Firstly, because our trait measurements were consistent with the previous descriptions of species, but because, in addition to the reliability, it allows for a numeric quantification of exploration-type related traits and melanisation degree which was lacking in previous categorical classifications. Furthermore, the analyses showed, as expected, an effect of trace element contamination on the functional traits of ECM fungal communities.

6.4.1. Effect of contamination on ECM fungal community diversity and structure

Soil trace element contamination had no effect on ECM fungal richness. This fact has to be discussed due to the inconsistency of previous results. Some authors did find a negative impact of heavy metal contamination on ECM fungal diversity (Huang et al., 2014; Ruotsalanien et al., 2009; Staudenrausch et al., 2005). In contrast, other studies missed such an effect, in agreement with our results (see Hui et al., 2011; Op de Beeck et al., 2015). In our case, the relatively young age of the trees, all of them planted only 17 years ago, could increase the chances that stochastic effects, i.e. priority effects, were acting on the community assembly of the ECM fungal communities. This fact would explain two results: on one hand, the relatively low ECM species richness (average of 3.8 species per tree) in comparison with previous studies in near mature Mediterranean forests (evergreen *Quercus suber*) which averaged 6.3 species per tree (Aponte et al., 2010). This trend is in agreement with the known increase in ECM species richness during ecosystem development as observed by Visser (1995) or Wallander et al. (2010). On the other hand, the effect of soil background variables and trace element contamination on the ECM fungal community composition was relatively low (a small percentage of variation in species composition was explained by these variables). This is consistent with a primary successional scenario where stochastic processes such as dispersal and/or priority effects drive the community assembly (Jumpponen, 2003; Kennedy et al., 2009; Peay and Bruns, 2014) and thus blur the deterministic effects caused by soil factors, i.e. the proportion of

community composition explained by the soil environment or its effect on species richness. Indeed, although low as well, a certain proportion of the variation of the OTU community composition was found to depend on the spatial position, which is a sign of a stochastic process influencing community assembly. On the other hand, other environmental factors not measured in this study, such as the relative influence of the seasonal river floods on different sites, could be responsible for the proportion of unexplained variance in community composition.

Despite the variance explained by soil factors being limited, soil background variables and trace elements explained a similar proportion of the variation in species composition. Previous studies of ECM fungal communities have shown the important influence of nutrient-related variables, such as total C or organic C in soil, in the determination of ECM fungal community composition (Twieg et al., 2009). In our study, the two most frequent ECM species, *H. cavipes* and *T. terrestris*, were related to two independent abiotic gradients: *H. cavipes* to a trace element concentration gradient, and *T. terrestris* to a gradient in Ca concentration (likely related to the $CaCO_3$ and pH). This fact would explain why the variance in community composition was equally explained for each group of variables, as each of these groups explains the presence of one of the two most abundant ECM fungal species. Indeed, this result resembles the results by Op de Beeck et al. (2015) who also found that communities of ECM fungi were driven according to two environmental gradients: one responding to heavy metal contamination levels and the other driven by Fe, Mn, Mg and K.

6.4.2. Effect of contamination on mean fungal trait values

The effect of contamination was visible both in terms of the mean trait values of communities and the trait similarity across species in communities. Both rhizomorph and emanating hyphae frequency were found to be negatively associated with the concentration of some trace elements, which indicates a suppressive effect of the contamination on extramatrical mycelium growth. This effect has previously been found in controlled experiments, and varies across ECM fungal species (Qi et al., 2016). At the same time, the recorded patterns for the exploration-type related traits, particularly the relationship between emanating hyphae and total C, are also highly congruent with the

known variation of exploration type in response to changes in N sources in the soil, i.e. a change from inorganic to organic N sources will reduce the development of extramatrical mycelium (Hobbie and Agerer, 2010; Lilleskov et al., 2002; 2011). Previous studies have pointed out the capacity of melanin to biosorb Cu and reduce its environmental toxicity (Gadd and Rome, 1988), and that dark Ascomycota species usually are more resistant to heavy metal contamination than Basidiomycota (Likar and Regvar, 2013). The hypothesis that the degree of melanisation would increase with heavy metal concentration has to be rejected for this dataset since we did not record an increase in the black colour of ECM fungi present in contaminated sites compared with non-contaminated ones. The relationship between black colour of ECM fungal species and $CaCO_3$ could be the result of other biochemical interactions since melanin seems to be involved in the Ca^{2+} regulation of the cells (Bush et al., 2007). In the present study, the variation in CWM fungal traits explained by trace element concentrations doubled the variation explained by soil background variables. These effects were also independent of the spatial distribution of the samples, excluding any potential site effect in the results. This fact, together with the smaller overall variance explained in the case of the OTU matrix, highlights the interest of this trait-based approach to explain the consequences of trace element contamination on ECM fungal communities.

6.4.3. Ecological processes driving ECM fungal community assembly

The trait dispersion of species within communities was driven mainly by soil contamination and not by the nutrient status of the soil. The increase in Cd concentrations made species in ECM fungal communities become more similar in terms of presence of rhizomorphs. This reveals the potential environmental filtering that heavy metal contamination can have on the trait composition of ECM fungal communities. While species richness was similar across the studied sites, the increase in trait convergence indicates a reduction in the functional diversity of the community (Bässler et al., 2015) in response to soil contamination. Although we also found an average reduction in the emanating hyphae with increasing contamination levels, the tendency, marginally significant, with increasing contamination was a divergence in the frequency of emanating hyphae produced by species in the same community. This is not in agreement with an environmental filtering, as suggested for rhizomorphs, but could indicate that

competition between species is selecting species that differ in this trait. This could be explained by an interaction between the two traits: once the community has been filtered according to the production of rhizomorphs, the remaining subset of species is selected against biotic interactions, i.e. competition, as observed for example by Ingram and Shurin (2009).

The consequences of the reduction in the functional diversity of ECM fungal communities for plant and ecosystem functioning might depend on the specific traits affected. For ECM fungi it is known that the decomposition rate of their biomass is very dependent on melanin content and hyphal architecture (i.e. hydrophobic rhizomorphs versus hydrophilic feeder hyphae, Fernandez et al., 2016), which thus influences C storage in soil (Clemmensen et al., 2015). Additionally, these two traits also have an important role in water stress alleviation for plants (Fernandez and Koide, 2013), which may have important consequences for host fitness, particularly in Mediterranean environments.

6.5. Conclusions

In this study, we demonstrated that ECM functional traits correlated better with soil contamination than fungal taxonomic diversity or community structure. Thus, adding trait-based approaches to the description of ECM fungal communities facilitates a better understanding of the potential consequences of environmental degradation on ecosystem functioning. The often contradictory results of the effect of environmental impact on ECM fungal communities at the species level, both in terms of community compositions and taxonomic diversity, can be overcome by these functional approaches. However, more research is needed to show how the community trait changes influences the functionality of ecosystems.

6.6. Bibliography

Agerer, R. 2001. Exploration types of ectomycorrhizae: a proposal to classify ectomycorrhizal mycelial systems according to their patterns of differentiation and putative ecological importance. Mycorrhiza, 11, 107–114.

Agerer, R. 2006. Fungal relationships and structural identity of their ectomycorrhizae. Mycological Progress, 5, 67-107.

Aguilar-Trigueros, C.A., Powell, J.R., Anderson, I.C., Antonovics, J., Rillig, M.C. 2014. Ecological understanding of root-infecting fungi using trait-based approaches. Trends in Plant Science, 19, 432–438.

Albornoz, F.E., Teste, F.P., Lambers, H., Bunce, M., Murray, D.C., White, N.E., Laliberté, E. 2016. Changes in ectomycorrhizal fungal community composition and declining diversity along a 2-million-year soil chronosequence. Molecular Ecology, 25, 4919–4929.

Aponte, C., García, L.V., Marañón, T., Gardes, M. 2010. Indirect host effect on ectomycorrhizal fungi: leaf fall and litter quality explain changes in fungal communities on the roots of co-occurring Mediterranean oaks. Soil Biology and Biochemistry, 42, 788-796.

Bardgett, R.D., Mommer, L., De Vries, F.T. 2014. Going underground: root traits as drivers of ecosystem processes. Trends in Ecology and Evolution, 29, 692–699.

Bässler, C., Heilmann-Clausen, J., Karasch, P., Brandl, R., Halbwachs, H. 2015. Ectomycorrhizal fungi have larger fruit bodies than saprotrophic fungi. Fungal Ecology, 17, 205-212.

Benjamini, Y., Yekuteli, D. 2001. The control of the false discovery rate in multiple testing under dependency. The Annals of Statistics, 29, 1165-1188.

Blanchet, F.G., Legendre, P., Borcard, D. 2008. Forward selection of explanatory variables. Ecology, 89, 2623-2632.

Bray, R.H., Kurtz, L.T. 1945. Determination of total, organic and available forms of phosphorus in soils. Soil Science, 59, 39-45.

Bu, W., Schmid, B., Liu, X., Li, Y., Härdtle, W., von Oheimb, G., Liang, Y., Sun, Z., Huang, Y. et al. 2017. Inter- and intraspecific variation in specific root length drives aboveground biodiversity effects in young experimental forest stands. Journal of Plant Ecology, 10, 158-169.

Burgos, P., Madejón, E., Pérez de Mora, A., Cabrera, F. 2008. Horizontal and vertical variability of soil properties in a trace element contaminated area. International Journal of Applied Earth Observation and Geoinformation, 10, 11-25.

Bush, W.D., Simon, J.D. 2007. Quantification of Ca^{2+} binding to melanin supports the hypothesis that melanosomes serve as functional role in regulating calcium homeostasis. Pigment Cell Research, 20, 134-139.

Cabrera, F., Clemente, L., Díaz Barrientos, E., López, R., Murillo, J.M. 1999. Heavy metal pollution of soils affected by the Guadiamar toxic flood. Science of the Total Environment, 242, 117-129.

Cadotte, M.W., Carscadden, K., Mirotchnick, N. 2011. Beyond species: functional diversity and the maintenance of ecological processes and services. Journal of Applied Ecology, 48, 1079–1087.

Clemmensen, K.E., Bahr, A., Ovaskainen, O., Dahlberg, A., Ekblad, A., Wallander, H., Stenlid, J., Finlay, R.D., Wardle, D.A., Lindahl, B.D. 2013. Roots and associated fungi drive long-term carbon sequestration in boreal forest. Science, 339, 1615–1618.

Clemmensen, K.E., Finlay, R.D., Dahlberg, A., Stenlid, J., Wardle, D.A. 2015. Carbon sequestration is related to mycorrhizal fungal community shifts during long-term succession in boreal forests. New Phytologist, 205, 1525–1536.

Cordero, R.J. 2017. Melanin for space travel radioprotection. Environmental Microbiology, 19: 2529-2532.

Courty, P.E., Munoz, F., Selosse, M.A., Duchemin, M., Criquet, S., Ziarelli, F., Buée, M., Plassard, C., Taudière, A., Garbaye, J., et al. 2016. Into the functional ecology of ectomycorrhizal communities: environmental filtering of enzymatic activities. Journal of Ecology, 104, 1585–1598.

Crowther, T.W., Maynard, D.S., Crowther, T.R., Peccia, J., Smith, J.R., Bradford, M.A. 2014. Untangling the fungal niche: the trait-based approach. Frontiers in Microbiology, 5, 579.

Davey, M., Blaalid, R., Vik, U., Carlsen, T., Kauserud, H., Eidesen, P.B. 2015. Primary succession of *Bistorta vivipara* (L.) Delabre (Polygonaceae) root-associated fungi mirrors plant succession in two glacial chronosequences. Environmental Microbiology, 17, 2777-2790.

Demolon, A., Leroux, D. 1952. Guide pour l'etude experimental des sols. Gauthier-Villars, Paris.

De la Riva, E.G., Marañón, T., Pérez-Ramos, I.M., Navarro-Fernández, C.M., Olmo, M., Villar, R. 2017. Root traits across environmental gradients in Mediterranean woody communities: are they aligned along the root economics spectrum? Plant and Soil, 424, 35–48.

Díaz, S., Cabido, M. 2001. Vive la difference: plant functional diversity matters to ecosystem processes. Trends in Ecology and Evolution, 16, 646–655.

Díaz, S., Lavorel, S., de Bello, F., Quétier, F., Grigulis, K., Robson, T.M. 2007. Incorporating plant functional diversity effects in ecosystem service assessments. Proceedings of the National Academy of Sciences of the United States of America, 104, 20684–20689.

Domínguez, M.T., Marañón, T., Murillo, J.M., Schulin, R., Robinson, B.H. 2008. Trace element accumulation in woody plants of the Guadiamar valley, SW Spain: a large scale phytomanagement case study. Environmental Pollution, 152, 50-59.

Domínguez, M.T., Madejón, P., Marañón, T., Murillo, J.M. 2010. Afforestation of a trace-element polluted area in SW Spain: woody plant performance and trace element accumulation. European Journal of Forest Research, 129, 47–59.

Domínguez, M.T., Alegre, J.M., Madejón, P., Madejón, E., Burgos, P., Cabrera, F., Marañón, T., Murillo, J.M. 2016. River banks and channels as hotspots of soil pollution after large-scale remediation of a river basin. Geoderma, 261, 133-140.

Domínguez, M.T., Montiel-Rozas, M.M., Madejón, P., Díaz, M.J., Madejón, E. 2017. The potential of native species as bioenergy crops on trace-element contaminated Mediterranean lands. Science of the Total Environment, 590–591, 29–39.

Dray, S., Dufour, A.B. 2007. The ade4 package: implementing the duality diagram for ecologists. Journal of Statistical Software, 22, 1-20.

Dray, S., Legendre, P. 2008. Testing the species traits environment relationships: the fourth-corner problem revisited. Ecology, 89, 3400–3412.

Fernandez, C.W., Koide, R.T. 2014. Initial melanin and nitrogen concentrations control the decomposition of ectomycorrhizal fungal litter. Soil Biology and Biochemistry, 77, 150–157.

Fernandez, C.W., Koide, R.T. 2013. The function of melanin in the ectomycorrhizal fungus *Cenococcum geophilum* under water stress. Fungal Ecology, 6, 479–486.

Fernandez, C.W., Langley, J.A., Chapman, S., McCormack, M.L., Koide, R.T. 2016. The decomposition of ectomycorrhizal fungal necromass. Soil Biology and Biochemistry, 93, 38–49.

Gadd, G.M., de Rome, L., 1988. Biosorption of copper by fungal melanin. Applied Microbiology and Biotechnology 29, 610–617.

Galli, U., Schuepp, H., Brunold, C. 1994. Heavy-metal binding by mycorrhizal fungi. Physiologia Plantarum, 92, 364–368.

Gardes, M., Bruns, T.D. 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. Molecular Ecology, 2, 113–118.

Garnier, E., Navas, M.L., Grigulis, K. 2016. Plant functional diversity: Organism traits, community structure, and ecosystem properties. Oxford University Press, Oxford.

Gotelli, N.J. 2000. Null model analysis of species co-occurrence patterns. Ecology, 81, 2606–2621.

Götzenberger, L., de Bello, F., Brathen, K.A., Davison, J., Dubuis, A., Guisan, A., Leps, J., Lindborg, R., Moora, M., Partel, M., et al. 2012. Ecological assembly rules in plant communities-approaches, patterns and prospects. Biological Reviews, 87, 111–127.

Gould, I.J., Quinton, J.N., Weigelt, A., De Deyn, G.B., Bardgett, R.D., Seabloom, E. 2016. Plant diversity and root traits benefit physical properties key to soil function in grasslands. Ecology Letters, 19, 1140–1149.

Grimalt, J.O., Ferrer, M., Macpherson, E. 1999. The mine tailing accident in Aznalcóllar. The science of the Total Environment, 242, 3-11.

Gross, J. 2003. Variance inflation factors. R News, 3, 13–15.

Hobbie, E.A., Agerer, R. 2010. Nitrogen isotopes in ectomycorrhizal sporocarps correspond to belowground exploration types. Plant and Soil, 327, 71–83.

Huang, J., Nara, K., Lian, C., Zong, K., Peng, K., Xue, S., Shen, Z. 2012. Ectomycorrhizal fungal communities associated with Masson pine (*Pinus massoniana* Lamb.) in Pb-Zn mine sites of central south China. Mycorrhiza, 22, 589–602.

Huang, J., Nara, K., Zong, K., Wang, J., Xue, S., Peng, K., Shen, Z., Lian, C. 2014. Ectomycorrhizal fungal communities associated with Masson pine (*Pinus massoniana*) and white oak (*Quercus fabri*) in a manganese mining region in Hunan Province, China. Fungal Ecology, 9, 1-10.

Hui, N., Jumpponen, A., Niskanen, T., Liimatainen, K., Jones, K.L., Koivula, T., Romantschuk, M., Strömmer, R. 2011. EcM fungal community structure, but not diversity, altered in a Pb-contaminated shooting range in a boreal coniferous forest site in Southern Finland. FEMS Microbiology Ecology, 76, 121–132.

Ingram, T., Shurin, J.B. 2009. Trait-based assembly and phylogenetic structure in northeast Pacific rockfish assemblages. Ecology, 90, 2444–2453.

Jumpponen, A. 2003. Soil fungal community assembly in a primary successional glacier forefront ecosystem as inferred from rDNA sequence analyses. New Phytologist, 158, 569–578.

Katoh, K., Standley, D.M. 2013. MAFFT Multiple Sequence Alignment Software Version 7: improvements in performance and usability. Molecular Biology and Evolution, 30, 772-780.

Kembel, S.W., Cowan, P.D., Helmus, M.R., Cornwell, W.K., Morlon, H., Ackerly, D.D., Blomberg, S.P., Webb, C.O. 2010. Picante: R tools for integrating phylogenies and ecology. Bioinformatics, 26, 1463–1464.

Kennedy, P.G., Peay, K.G., Bruns, T.D. 2009. Root tip competition among ectomycorrhizal fungi: are priority effects a rule or an exception? Ecology, 90, 2098–2107.

Kjøller, R., Nilsson, L.O., Hansen, K., Schmidt, I.K., Vesterdal, L., Gundersen, P. 2012. Dramatic changes in ectomycorrhizal community composition, root tip abundance and mycelial production along a stand-scale nitrogen deposition gradient. New Phytologist, 194, 278–286.

Kleyer, M., Dray, S., de Bello, F., Lepš, J., Pakeman, R.J., Strauss, B., Thuiller, W., Lavorel, S. 2012. Assessing species and community functional responses to environmental gradients: which multivariate methods? Journal of Vegetation Science, 23, 805–821.

Kogej, T., Gorbushina, A.A., Gunde-Cimerman, N. 2006. Hypersaline conditions induce changes in cell-wall melanization and colony structure in a halophilic and a xerophilic black yeast species of the genus Trimmatostroma. Mycological Research, 110, 713–724.

Koide, R.T., Fernandez, C., Malcolm, G. 2014. Determining place and process: functional traits of ectomycorrhizal fungi that affect both community structure and ecosystem function. New Phytologist, 201, 433–439.

Kõljalg, U., Larsson, K.H., Abarenkov, K., Nilsson, R.H., Alexander, I.J., Eberhardt, U., Erland, S., Høiland, K., Kjøller, R., Larsson, E., et al. 2005. UNITE: a database providing web-based methods for the molecular identification of ectomycorrhizal fungi. New Phytologist, 166, 1063-1068.

Laliberté, E. 2016. Below-ground frontiers in trait-based plant ecology. New Phytologist, 213, 1597–1603.

Lavorel, S., Garnier, E. 2002. Predicting changes in community composition and ecosystem functioning from plant traits: revisiting the Holy Grail. Functional Ecology, 16, 545–556.

Lavorel, S., Storkey, J., Bardgett, R.D., de Bello, F., Berg, M.P., Le Roux, X., Moretti, M., Mulder, C., Pakeman, R.J., Díaz, S. et al. 2013. A novel framework for linking functional diversity of plants with other trophic levels for the quantification of ecosystem services. Journal of Vegetation Science, 24, 942–948.

Legendre, P., Gallagher, E. 2001. Ecologically meaningful transformations for ordination of species data. Oecologia, 129, 271–280.

Legendre, P., Legendre, L. 1998. Numerical Ecology. Amsterdam, Netherlands: Elsevier Science.

Legendre, P., Galzin, R., Harmelin-Vivien, M.L. 1997. Relating behavior to habitat: solutions to the fourth-corner problem. Ecology, 78, 547–562.

Lepš, J., de Bello, F., Šmilauer, P., Doležal, J. 2011. Community trait response to environment: disentangling species turnover vs intraspecific trait variability effects. Ecography, 34, 856–863.

Likar, M., Regvar, M. 2013. Isolates of dark septate endophytes reduce metal uptake and improve physiology of *Salix caprea* L. Plant and Soil, 370, 593-604.

Lilleskov, E.A., Hobbie, E.A., Fahey, T.J. 2002. Ectomycorrhizal fungal taxa differing in response to nitrogen deposition also differ in pure culture organic nitrogen use and natural abundance of nitrogen isotopes. New Phytologist, 154, 219–231.

Lilleskov, E.A., Hobbie, E.A., Horton, T.R. 2011. Conservation of ectomycorrhizal fungi: exploring the linkages between functional and taxonomic responses to anthropogenic N deposition. Fungal Ecology, 4, 174–183.

López-García, Á., Azcón-Aguilar, C., Barea, J.M. 2014. The interactions between plant life form and fungal traits of arbuscular mycorrhizal fungi determine the symbiotic community. Oecologia, 176, 1075–1086.

Maynard, D.G., Kalra, Y.P., Crumbaugh, J.A. 2007. Nitrate and exchangeable ammonium nitrogen. In: Carter, M.R., Gregorich, E.G., eds. Soil sampling and methods of analysis, 2nd ed., Boca Raton, USA: CRC Press, Taylor and Francis Group, 71-80.

Moeller, H.V., Peay, K.G., Fukami, T. 2014. Ectomycorrhizal fungal traits reflect environmental conditions along a coastal California edaphic gradient. FEMS Microbiology Ecology, 87, 797–806.

Mulder, C., Cohen, J.E., Setälä, H., Bloem, J., Breure, A.M. 2005. Bacterial traits, organism mass, and numerical abundance in the detrital soil food web of Dutch agricultural grasslands. Ecology Letters, 8, 80–90.

Oksanen, J., Blanchet, F.G., Kindt, R. 2012. Vegan: Community ecology package.

Olsen, S.R., Cole, C.V., Watanabe, F.S., Dean, L.A. 1954. Estimation of available phosphorus in soils by extraction with sodium bicarbonate. USDA Circular 939. Washington DC: US gov Print Office.

Op De Beeck, M., Ruytinx, J., Smits, M.M., Vangronsveld, J., Colpaert, J.V., Rineau, F. 2015. Belowground fungal communities in pioneer Scots pine stands growing on heavy metal polluted and non-polluted soils. Soil Biology and Biochemistry, 86, 58–66.

Pawlowska, T.E., Charvat, I. 2004. Heavy-metal stress and developmental patterns of arbuscular mycorrhizal fungi. Applied and Environmental Microbiology, 70, 6643–6649.

Peay, K.G., Bruns, T.D. 2014. Spore dispersal of basidiomycete fungi at the landscape scale is driven by stochastic and deterministic processes and generates variability in plant-fungal interactions. New Phytologist, 204, 180–191.

Pelosi, C., Pey, B., Hedde, M., Caro, G., Capowiez, Y., Guernion, M., Peignée, J., Pirond, D., Bertrandfg, M., Cluzeaud, D. 2014. Reducing tillage in cultivated fields increases earthworm functional diversity. Applied Soil Ecology, 83, 79–87.

Qi, Y., Zhao, N., Liu, J., Huang, J. 2016. Biochemical responses of ten ectomycorrhizal fungal isolates to manganese. Water, Air and Soil Pollution, 227, 477-487.

Rosas, A.L., Casadevall, A. 2001. Melanization decreases the susceptibility of *Cryptococcus neoformans* to enzymatic degradation. Mycopathologia, 151, 53-56.

Ruotsalanien, A.L., Markkola, A.M., Kozlov, M.V. 2009. Mycorrhizal colonisation of mountain birch (*Betula pubescens* ssp. czerepanovii) along three environmental gradients: does life in harsh environments alter plant–fungal relationships? Environmental Monitoring Assessment, 148, 215-232.

Santorufo, L., Cortet, J., Nahmani, J., Pernin, C., Salmon, S., Pernot, A., Morel, J.L., Maisto, G. 2015. Responses of functional and taxonomic collembolan community structure to site management in Mediterranean urban and surrounding areas. European Journal of Soil Biology, 70, 46–57.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., Weber, C.F. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied and Environmental Microbiology, 75, 7537–41.

Sousa, N.R., Ramos, M.A., Marques, A.P.G.C, Castro, P.M.L. 2014. A genotype dependent-response to cadmium contamination in soil is displayed by *Pinus pinaster* in symbiosis with different mycorrhizal fungi. Applied Soil Ecology, 76, 7–13.

Staudenrausch, S., Kaldorf, M., Renker, C., Luis, P., Buscot, F. 2005. Diversity of the ectomycorrhiza community at a uranium mining heap. Biology and Fertility of Soils, 41, 439–446.

Suz, L.M., Barsoum, N., Benham, S., Dietrich, H.P., Fetzer, K.D., Fischer, R., García, P., Gehrman, J., Kristöfel, F., Manninger, M. et al. 2014. Environmental drivers of ectomycorrhizal communities in Europe's temperate oak forests. Molecular Ecology, 23, 5628–5644.

Tedersoo, L., Naadel, T., Bahram, M., Pritsch, K., Buegger, F., Leal, M., Kõljalg, U., Põldmaa, K. 2012. Enzymatic activities and stable isotope patterns of ectomycorrhizal fungi in relation to phylogeny and exploration types in an afrotropical rain forest. New Phytologist, 195, 832–843.

ter Braak, C.J.F., Schaffers, A.P. 2012. Co-Correspondence Analysis: a new ordination method to relate two community compositions. Ecology, 85, 834–846.

Treseder, K.K., Lennon, J.T. 2015. Fungal traits that drive ecosystem dynamics on land. Microbiology and Molecular Biology Reviews, 79, 243–262.

Tunlid, A., Floudas, D., Koide, R.T., Rineau, F. 2017. Soil organic matter decomposition mechanisms in ectomycorrhizal fungi. In: Martin F, ed. Molecular Mycorrhizal Symbiosis. Hoboken, NJ, USA: John Wiley & Sons, Inc.

Twieg, B.D., Durall, D.M., Simard, S.W., Jones, M.D. 2009. Influence of soil nutrients on ectomycorrhizal communities in a chronosequence of mixed temperate forests. Mycorrhiza, 19, 305– 316.

van der Heijden, M.G.A., Martin, F.M., Selosse, M.A., Sanders, I.R. 2015. Mycorrhizal ecology and evolution: the past, the present, and the future. New Phytologist, 205, 1406–1423.

Visser, S. 1995. Ectomycorrhizal fungal succession in jack pine stands following wildfire. New Phytologist, 129, 389–401.

Wallander, H., Johansson, U., Sterkenburg, E., Durling, M.B., Lindahl, B.D. 2010. Production of ectomycorrhizal mycelium peaks during canopy closure in Norway spruce forests. New Phytologist, 187, 1124–1134.

White, T.J., Bruns, S., Lee, S., Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H, Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, London, pp. 315-322.

6.7. Supplementary material

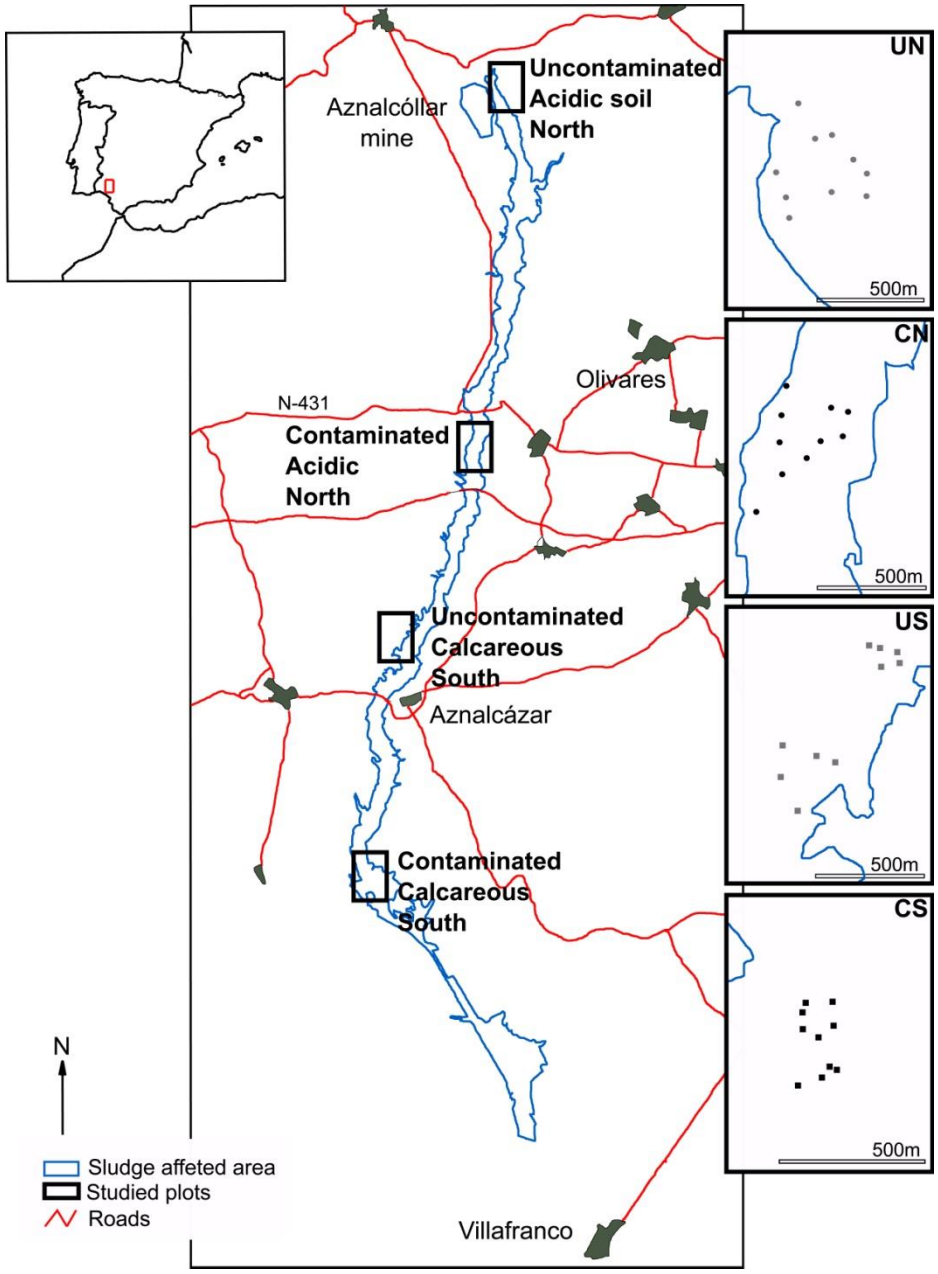


Figure S6.1 Map of the study area and location of plots.

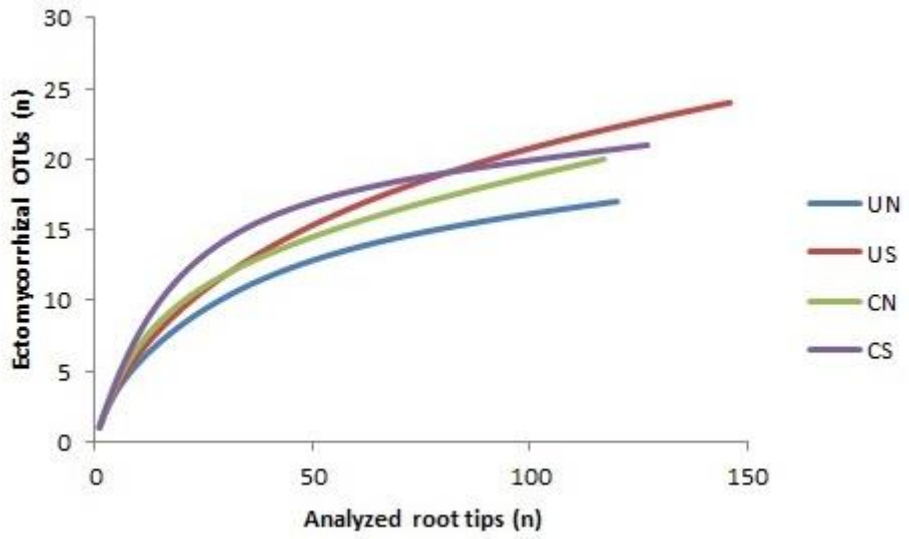


Figure S6.2 Rarefaction analysis of OTU distribution in the analyzed ectomycorrhizal root tips from Guadiamar river valley (SW Spain). Contaminated North (CN), Contaminated South (CS), Uncontaminated North (UN), Uncontaminated South (US).

Table S6.1 Overall distribution of texture components in the sampled plots (data from Domínguez pers. comm.) and soil type classification (according to Clemente et al. 2000) in the four sample sites in Guadiamar river valley (SW Spain). Geographic locations of specific sampled trees. Contaminated north (CN) and south (CS), uncontaminated north (UN) and south (US).

	CN	**CS**	**UN**	**US**
Coarse sand (%)	30.1	24.5	31.6	24.8
Fine sand (%)	21.6	15.2	16.2	27.2
Silt (%)	27.8	33.3	31.2	24.1
Clay (%)	20.4	27	21.1	22.7
Soil type	Typic/Aquic Xerofluvent	Aquic Haploxeralf	Typic Xerofluvent	Typic Rhodoxeralf/ Typic Haploxeralf
UTM coordinates of sampled trees				
	37.386733,-6.226050	37.242796,-6.262997	37.501699,-6.223200	37.326128,-6.254079
	37.385683,-6.226283	37.242426,-6.263540	37.500837,-6.222986	37.326017,-6.253461
	37.384788,-6.228140	37.243197,-6.264157	37.501934,-6.220785	37.325835,-6.252575
	37.385500,-6.227400	37.242692,-6.264152	37.501747,-6.218971	37.325364,-6.252395
	37.387800,-6.229283	37.241216,-6.263381	37.502676,-6.218921	37.321194,-6.255822
	37.386588,-6.229400	37.240979,-6.264334	37.503267,-6.219647	37.321470,-6.256862
	37.385405,-6.229497	37.241460,-6.262851	37.504298,-6.220750	37.321916,-6.258642
	37.384155,-6.229326	37.241546,-6.263120	37.504149,-6.221652	37.320483,-6.258804
	37.382667,-6.229817	37.243488,-6.264055	37.505631,-6.222518	37.319079,-6.257846

Table S6.2 Species list, number of root tips identified in each plot (Contaminated North - CN, Contaminated South - CS, Uncontaminated North - UN, Uncontaminated South - US), number of trees in which they were detected (Occurence), blast results against the UNITE database and Species Hypothesis (SH) (only matches higher than 97% are shown).

Phylum	Family	Species	CN	CS	UN	US	Occurrence	Closest match (Acc. No.)	Identity (%)	Species Hypothesis (UNITE)
Ascomycota										
	Gloniaceae	*Cenococcum geophyllum*			4	10	3	Uncultured ectomycorrhiza (*Cenococcum geophilum*) (AY299214)	99	SH214459.07FU
	Pyronemataceae	*Geopora cervina*		9			2	Uncultured *Geopora* (GU327416)	99	SH213655.07FU
	Pyronemataceae	*Geopora* sp.		1			1	*Geopora* sp. (UDB011007)	97	SH213666.07FU
	Pezizaceae	*Peziza michelii*		5		1	3	*Peziza michelii* (JF908553)	98	SH218195.07FU
	Pezizaceae	*Peziza* sp.	1				1	*Peziza* sp. (KP311474)	99	SH189857.07FU
	Pyronemataceae	*Pustularia* sp.	3	8		2	4	Uncultured Ascomycete (EU557319)	99	SH222141.07FU
	Pyronemataceae	Pyronemataceae sp. 1			1		1	Uncultured fungus (JF927116)	93	SH213666.07FU
	Pyronemataceae	Pyronemataceae sp. 2		4			1	Uncultured fungus (KM247654)	99	-
	Pyronemataceae	Pyronemataceae sp. 3				4	1	Uncultured ectomycorrhizal fungus (FJ008039)	99	SH025866.07FU
	Pyronemataceae	*Trichophaeae* sp.	2	7		4	5	Uncultured Pyronemataceae sp. (HM370456)	97	SH215396.07FU
	Tuberaceae	*Tuber oligospermum*				1	1	*Tuber oligospermum* (FM205504)	97	SH188863.07FU
	Tuberaceae	*Tuber* sp. 1		1			2	*Tuber* sp. (KC517481)	95	-
	Tuberaceae	*Tuber* sp. 2		3			1	Uncultured *Tuber* (HQ204754)	96	-
	Tuberaceae	*Tuberaceae* sp. 1	1		7		1	Uncultured ectomycorrhizal fungus (HM057200)	92	SH185378.07FU

Phylum	Family	Species	CN	CS	UN	US	Occurrence	Closest match (Acc. No.)	Identity (%)	Species Hypothesis (UNITE)
Basidiomycota										
	Diplocystidiaceae	*Astraeus hygrometricus*	3		5	1	4	*Astraeus hygrometricus* (HG000293)	99	SH190454.07FU
	Cortinariaceae	*Cortinarius belleri*			5		2	*Cortinarius belleri* (AY669685)	99	SH188471.07FU
	Cortinariaceae	*Cortinarius subbalaustinus*				6	3	Uncultured *Cortinarius* (GU246986)	99	SH188517.07FU
	Cortinariaceae	*Cortinarius subturibulosus*			3	24	5	Uncultured mycorrhizal fungus (FJ897182)	100	SH188545.07FU
	Entolomataceae	*Entoloma inusitatum*	7				3	Uncultured Entolomaceae (FJ210729)	99	SH181020.07FU
	Cortinariaceae	*Hebeloma cavipes*	10		26	45	19	*Hebeloma cavipes* (KT225477)	100	SH215994.07FU
	Cortinariaceae	*Hebeloma cistophilum*				3	1	Uncultured fungus clone (HQ625447)	99	SH218875.07FU
	Strophariaceae	*Hymenogaster griseus*		1			1	*Hymenogaster griseus* (AF325636)	99	SH218859.07FU
	Inocybaceae	*Inocybe curvipes*	1		3		3	Inocybe cf. curvipes (KT275613)	97	SH201231.07FU
	Inocybaceae	*Inocybe griseovelata*		6			2	Inocybe griseovelata (JF908237)	97	SH176687.07FU
	Inocybaceae	*Inocybe jacobi*	1				1	Inocybe jacobi (HQ604812)	99	SH211892.07FU
	Inocybaceae	*Inocybe praetervisa*				1	1	Inocybe sp. (KM576438)	98	SH212066.07FU
	Inocybaceae	*Inocybe squamata*				1	1	*Inocybe squamata* (FJ904136)	99	SH222043.07FU
	Hydnangiaceae	*Laccaria laccata*	4		3		3	*Laccaria laccata* (KM067883)	100	SH220959.07FU

Phylum	Family	Species	CN	CS	UN	US	Occurrence	Closest match (Acc. No.)	Identity (%)	Species Hypothesis (UNITE)
Basidiomycota										
	Russulaceae	*Lactarius* sp. 1	1				1	*Lactarius atlanticus* (KR025612)	96	-
	Russulaceae	*Lactarius* sp. 2	1				1	*Lactarius atlanticus* (KP420216)	95	-
	Paxillaceae	*Melanogaster vittadinii*				1	1	Melanogaster vittadinii (AJ555525)	97	SH182656.07FU
	Sclerodermataceae	*Pisolithus arhizus*			1		1	*Pisolithus arhizus* (FR748128)	98	SH177625.07FU
	Sclerodermataceae	*Pisolithus tinctorius*			5	3	2	*Pisolithus tinctorius* (HE578142)	99	SH177623.07FU
	Russulaceae	*Russula amoenolens*	19		1	2	5	Russulaceae (KT334781)	99	SH220816.07FU
	Russulaceae	*Russula ilicis*			9		1	Uncultured Russulaceae (HQ330996)	99	SH180269.07FU
	Russulaceae	*Russula insignis*		9			3	*Russula insignis* (AY061700)	98	SH220848.07FU
	Russulaceae	*Russula praetervisa*	10	5	2	16	5	Uncultured *Russula* (FR852096)	97	SH202443.07FU
	Russulaceae	*Russula* sp.				1	1	Uncultured *Russula* (KT334781)	95	-
	Sclerodermataceae	*Scleroderma cepa*	4				1	*Scleroderma laeve* (KP004932)	99	SH182463.07FU
	Sclerodermataceae	*Scleroderma meridionale*			1		1	*Scleroderma meridionale* (HF933239)	100	SH186878.07FU
	Sclerodermataceae	*Scleroderma* sp. 1			1		1	Uncultured fungus (FM999606)	95	SH179758.07FU
	Sclerodermataceae	*Scleroderma verrucosum*	13	3		1	6	Uncultured fungus (KM247623)	99	SH182460.07FU
	Thelephoraceae	*Thelephora terrestris*	14		42	5	12	Uncultured *Thelephora terrestris* (KF007266)	99	SH184510.07FU
	Thelephoraceae	*Tomentella castanea*	20				1	*Tomentella* cf. *sublilacina* (KU376404)	100	SH184517.07FU

Phylum	Family	Species	CN	CS	UN	US	Occurrence	Closest match (Acc. No.)	Identity (%)	Species Hypothesis (UNITE)
Basidiomycota										
	Thelephoraceae	*Tomentella ferruginea*		8			1	Uncultured fungus clone (KM247776)	99	SH184518.07FU
	Thelephoraceae	*Tomentella lilacinogrisea*				3	1	Uncultured fungus clone (KF297246)	99	SH178628.07FU
	Thelephoraceae	*Tomentella* sp. 1		1			1	Uncultured fungus clone (KM247736)	99	-
	Thelephoraceae	*Tomentella* sp. 10		1			1	Uncultured *Tomentella* (FJ197002)	96	-
	Thelephoraceae	*Tomentella* sp. 2				7	1	Uncultured fungus clone (KM247732)	99	SH177905.07FU
	Thelephoraceae	*Tomentella* sp. 3				1	1	Uncultured *Tomentella* (FJ210771)	99	SH184642.07FU
	Thelephoraceae	*Tomentella* sp. 4		4			1	Uncultured *Tomentella* (JX630358)	97	SH184626.07FU
	Thelephoraceae	*Tomentella* sp. 5		10			1	Uncultured *Tomentella* (LC013836)	98	-
	Thelephoraceae	*Tomentella* sp. 6	1	15			3	Uncultured fungus (FN397409)	99	SH177879.07FU
	Thelephoraceae	*Tomentella* sp. 8	1	13			2	Uncultured *Tomentella* (FR852207)	99	SH002639.07FU
	Thelephoraceae	*Tomentella* sp. 9				1	1	Uncultured *Tomentella* (KC840637)	99	SH177797.07FU

Table S6.3 Fungal trait measurements in the current study (Guadiamar river valley, SW Spain) and comparison with records of Deemy database (http://www.deemy.de). The experimental observations are expressed in term of frequency (percentage) of number of root tips exhibiting either emanating hyphae or rhizomorphs, and the black color content (0-100) of root tips for melanisation. The records of species in this study are compared with the records of the same species in Deemy database (01-10-2017) when available (marked with asterisk). When the species was not recorded in Deemy, records from species of the same genera were displayed. The percentage of records showing different the different categories was shown. NA: absence of data; Distance Exploration types: Contact, Short, Medium mat, Medium fringe and Medium smooth (Agerer 2001, 2006); Emanating hyphae and rhizomorphs: Absent, Infrequent and Abundant. The n column is the number of root tips found for each species.

				Experimental observations			Deemy database		
Phylum	Family	Species	n	Emanating hyphae	Rhizomorphs	Melanisation	Exploration type	Emanating hyphae	Rhizomorph presence
Ascomycota									
	Gloniaceae	*Cenococcum geophyllum**	14	100	0	90.5	Short	Abundant	Absent
	Pyronemataceae	*Geopora cervina*	9	11.1	22.2	81.1	NA	NA	NA
	Pyronemataceae	*Geopora* sp.	1	100	0	84	NA	NA	NA
	Pezizaceae	*Peziza michelii*	6	33.3	16.7	82.1	NA	NA	NA
	Pezizaceae	*Peziza* sp.	1	0	0	87.7	NA	NA	NA
	Pyronemataceae	*Pustularia* sp.	13	53.8	0	76.2	NA	NA	NA
	Pyronemataceae	Pyronemataceae sp. 1	1	0	0	80.3	NA	NA	NA
	Pyronemataceae	Pyronemataceae sp. 2	4	75	0	85.7	NA	NA	NA
	Pyronemataceae	Pyronemataceae sp. 3	4	75	0	87.6	NA	NA	NA
	Pyronemataceae	*Trichophaeae* sp.	13	84.6	7.7	83.2	NA	NA	NA
	Tuberaceae	*Tuber oligospermum*	1	100	100	84.7	Short	Abundant 26.1/ Infrequent 52.2/ Absent 21.7	Infrequent 4.4/ Absent 95.6
	Tuberaceae	*Tuber* sp. 1	1	100	0	64	Short	Abundant 26.1/ Infrequent 52.2/ Absent 21.7	Infrequent 4.4/ Absent 95.6

Phylum	Family	Species	n	Experimental observations			Deemy database		
				Emanating hyphae	Rhizomorphs	Melanisation	Exploration type	Emanating hyphae	Rhizomorph presence
	Tuberaceae	*Tuber* sp. 2	3	0	0	84.7	Short	Abundant 26.1/ Infrequent 52.2/ Absent 21.7	Infrequent 4.4/ Absent 95.6
	Tuberaceae	*Tuberaceae* sp. 1	8	12.5	0	77	NA	NA	NA
Basidiomycota									
	Diplocystidiaceae	*Astraeus hygrometricus*	9	44.4	44.4	79.3	NA	NA	NA
	Cortinariaceae	*Cortinarius belleri**	5	100	0	64.6	Medium fringe 96.2/ Medium mat 3.8	Abundant 65.4/ Infrequent 19.2	Abundant 80.8/ Infrequent 11.5
	Cortinariaceae	*Cortinarius subbalaustinus**	6	66.6	66.6	64.8	Medium fringe 96.2/ Medium mat 3.8	Abundant 65.4/ Infrequent 19.2	Abundant 80.8/ Infrequent 11.5
	Cortinariaceae	*Cortinarius subturibulosus**	27	96.3	25.9	72.9	Medium fringe 96.2/ Medium mat 3.8	Abundant 65.4/ Infrequent 19.2	Abundant 80.8/ Infrequent 11.5
	Entolomataceae	*Entoloma inusitatum**	7	42.9	0	68	Medium smooth	Abundant 33.3/ Infrequent 33.3/ Absent 33.3	Abundant 33/ Infrequent 66
	Cortinariaceae	*Hebeloma cavipes*	81	95.1	13.6	67.7	Short 87.5/ Medium 12.5	Abundant	Abundant 12.5/ Absent 87.5
	Cortinariaceae	*Hebeloma cistophilum*	3	100	0	69.7	Short 87.5/ Medium 12.5	Abundant	Abundant 12.5/ Absent 87.5
	Strophariaceae	*Hymenogaster griseus*	1	100	0	77.3	NA	NA	NA

				Experimental observations			Deemy database		
Phylum	Family	Species	n	Emanating hyphae	Rhizomorphs	Melanisation	Exploration type	Emanating hyphae	Rhizomorph presence
	Inocybaceae	*Inocybe curvipes*	4	50	0	64.1	Short	Abundant40/ Infrequent 60	Absent
	Inocybaceae	*Inocybe griseovelata*	6	66.7	0	71	Short	Abundant40/ Infrequent 60	Absent
	Inocybaceae	*Inocybe jacobi*	1	100	0	76	Short	Abundant40/ Infrequent 60	Absent
	Inocybaceae	*Inocybe praetervisa*	1	100	0	93.3	Short	Abundant40/ Infrequent 60	Absent
	Inocybaceae	*Inocybe squamata*	1	100	0	70.7	Short	Abundant40/ Infrequent 60	Absent
	Hydnangiaceae	*Laccaria laccata*	7	71.4	14.3	71.4	Medium smooth	Abundant 87.5/ Infrequent 62.5	Abundant 12.4/ Infrequent 37.5/ Absent 62.5
	Russulaceae	*Lactarius* sp. 1	1	0	0	79	Contact 35.7/ Medium smooth 64.3	Absent 56.4/ Infrequent 48.7	Abundant 2.4/ Infrequent 64.3/ Absent 33.3
	Russulaceae	*Lactarius* sp. 2	1	100	0	75.3	Contact 35.7/ Medium smooth 64.3	Absent 56.4/ Infrequent 48.7	Abundant 2.4/ Infrequent 64.3/ Absent 33.3
	Paxillaceae	*Melanogaster vittadinii*	1	100	100	85.5	Long	Infrequent	Abundant
	Sclerodermataceae	*Pisolithus arhizus*	1	0	0	77	NA	Infrequent	Abundant 50/ Infrequent 50
	Sclerodermataceae	*Pisolithus tinctorius**	8	75	37.5	78.2	NA	Infrequent	Infrequent
	Russulaceae	*Russula amoenolens**	22	36.4	4.5	70.7	Short 50/ Medium smooth 50	Infrequent	Infrequent
	Russulaceae	*Russula ilicis*	9	55.5	33.3	72.4	Contact 44.2 / Short 13.0/ Medium smooth 33.8	Absent 5.2/ Infrequent 84.4/ Abundant 2.6	Infrequent 44.2/ Absent 55.8

				Experimental observations			Deemy database		
Phylum	Family	Species	n	Emanating hyphae	Rhizomorphs	Melanisation	Exploration type	Emanating hyphae	Rhizomorph presence
	Russulaceae	*Russula insignis**	9	55.5	0	82.1	Short	Infrequent	Absent
	Russulaceae	*Russula praetervisa*	33	44.8	17.2	72.9	Contact 44.2 / Short 13.0/ Medium smooth 33.8	Absent 5.2/ Infrequent 84.4/ Abundant 2.6	Infrequent 44.2/ Absent 55.8
	Russulaceae	*Russula* sp.	1	100	100	71.3	Contact 44.2 / Short 13.0/ Medium smooth 33.8	Absent 5.2/ Infrequent 84.4/ Abundant 2.6	Infrequent 44.2/ Absent 55.8
	Sclerodermataceae	*Scleroderma cepa*	4	75	0	71.9	Long	Abundant	Abundant 75.0/ Infrequent 25.0
	Sclerodermataceae	*Scleroderma meridionale*	1	100	0	72.3	Long	Abundant	Abundant 75.0/ Infrequent 25.0
	Sclerodermataceae	*Scleroderma* sp. 1	1	100	0	69.7	Long	Abundant	Abundant 75.0/ Infrequent 25.0
	Sclerodermataceae	*Scleroderma verrucosum*	17	94.1	41.2	73.3	Long	Abundant	Abundant 75.0/ Infrequent 25.0
	Thelephoraceae	*Thelephora terrestris**	61	88.5	11.5	69.8	Medium smooth	Infrequent	Abundant 50.0/ Infrequent 50.0
	Thelephoraceae	*Tomentella castanea*	20	25	0	84.2	NA	Abundant 33.3/ Infrequent 66.6	Abundant 42.9/ Infrequent 57.1
	Thelephoraceae	*Tomentella ferruginea*	8	62.5	62.5	86.8	NA	Abundant 33.3/ Infrequent 66.6	Abundant 42.9/ Infrequent 57.1
	Thelephoraceae	*Tomentella lilacinogrisea*	3	100	66.7	83.9	NA	Abundant 33.3/ Infrequent 66.6	Abundant 42.9/ Infrequent 57.1
	Thelephoraceae	*Tomentella* sp. 1	1	100	0	86.3	NA	Abundant 33.3/ Infrequent 66.6	Abundant 42.9/ Infrequent 57.1

				Experimental observations			Deemy database		
Phylum	Family	Species	n	Emanating hyphae	Rhizomorphs	Melanisation	Exploration type	Emanating hyphae	Rhizomorph presence
	Thelephoraceae	*Tomentella* sp. 10	1	100	100	94.7	NA	Abundant 33.3/ Infrequent 66.6	Abundant 42.9/ Infrequent 57.1
	Thelephoraceae	*Tomentella* sp. 2	7	57.1	14.3	83.9	NA	Abundant 33.3/ Infrequent 66.6	Abundant 42.9/ Infrequent 57.1
	Thelephoraceae	*Tomentella* sp. 3	1	0	0	94.3	NA	Abundant 33.3/ Infrequent 66.6	Abundant 42.9/ Infrequent 57.1
	Thelephoraceae	*Tomentella* sp. 4	4	100	0	92.9	NA	Abundant 33.3/ Infrequent 66.6	Abundant 42.9/ Infrequent 57.1
	Thelephoraceae	*Tomentella* sp. 5	10	100	30	82.4	NA	Abundant 33.3/ Infrequent 66.6	Abundant 42.9/ Infrequent 57.1
	Thelephoraceae	*Tomentella* sp. 6	16	32.5	6.3	79.7	NA	Abundant 33.3/ Infrequent 66.6	Abundant 42.9/ Infrequent 57.1
	Thelephoraceae	*Tomentella* sp. 8	14	35.7	14.3	90.4	NA	Abundant 33.3/ Infrequent 66.6	Abundant 42.9/ Infrequent 57.1
	Thelephoraceae	*Tomentella* sp. 9	1	100	0	84	NA	Abundant 33.3/ Infrequent 66.6	Abundant 42.9/ Infrequent 57.1

Table S6.4 Forward selection of environmental variables for improving redundancy analysis of factors driving ectomycorrhizal community assembly in the Guadiamar river valley (SW Spain).

Species-based redundancy model	**Df**	**AIC**	**F**	**Pr(>F)**	
Trace elements					
S	1	-3.6989	2.3380	0.005	**
Ni	1	-4.3694	1.7278	0.005	**
Zn	1	-3.8652	2.1855	0.005	**
Cu	1	-3.2891	2.7170	0.005	**
Soil background variables					
Ca	1	-4.1287	2.9858	0.005	**
Organic C	1	-4.6259	2.5075	0.005	**
Total C	1	-4.0746	3.0383	0.005	**
CWM-based redundancy model	**Df**	**AIC**	**F**	**Pr(>F)**	
Trace elements					
Cu	1	200.87	6.8637	0.010	**
As	1	201.55	7.6055	0.010	**
Cd	1	203.38	9.7030	0.005	**
Soil background variables					
CaCO3	1	199.88	6.0131	0.010	**
Organic C	1	202.55	8.8758	0.005	**
Total C	1	204.94	11.6547	0.005	**
P	1	196.62	2.8140	0.090	.

6.7.1 Methods

ECM fungal trait determinations

The seven longest root fragments were selected from each root subsample. This made a total of 28 root fragments per tree. Root tips were selected randomly by choosing the extreme left of each root fragment. Each root tip was photographed in triplicates with a digital camera (Nikon DS-Fi1) fitted on a dissecting microscope. Two general pictures (25X magnification) on white and black background, and one detailed picture (100X magnification) on black background were taken, keeping light conditions at maximum and photograph exposition at 1/10s for the general pictures and 1/4s for the detailed one (Fig. S6.3; A-C). Three fungal traits – rhizomorphs, emanating hyphae and melanisation – were measured, as follows.

Rhizomorphs

The presence of rhizomorphs was recorded in the 25X magnified photographs. The presence of rhizomorphs was recorded for a root tip if a rhizomorph emerging from the cluster to which the selected root tip belongs was found (Fig. S6.3; D-H). This procedure was chosen because rhizomorphs are less frequent than individual emanating hypha in a random root tip; however, individual root tips often are part of a bigger cluster of root tips of the same individual fungus.

Emanating hyphae

Emanating hyphae was determined at 100X magnification on black background photographs. The presence of emanating hyphae was recorded when hyphae appeared continuous and homogeneously distributed in the root tip surface (Fig. S 6.3; L-N). However, when only individual, isolated, hyphae appeared, root tips were scored as having no emanating hyphae (Fig. S 6.3; I-K).

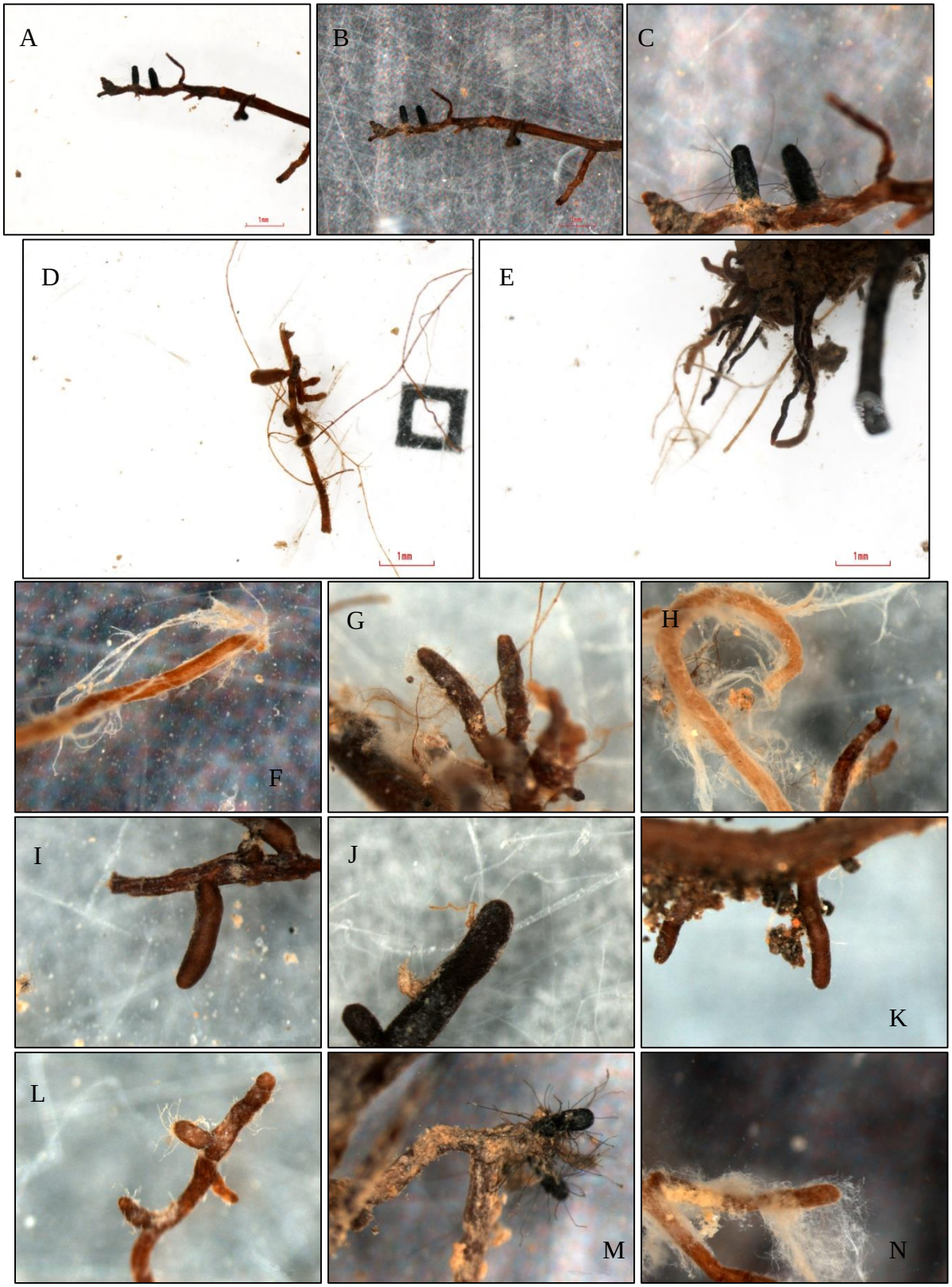


Figure S6.3 Examples of photographs showing root tips with different fungal traits. a-c) Cenococcum geophylum root tips at 25X magnification (a, b) and 100X magnification (c); d-e) clusters of root tips with associated rhizomorphs (25X magnification); f-h) detailed of root tips showing rhizomorphs (100X magnification); i-k) root tips with no emanating hyphae (100X magnification); l-n) root tips showing different morphologies

of emanating hyphae (100X magnification). The contrast of these pictures has been automatically improved to facilitate the visibility of fungal structures in this slide.

Melanisation

The colour of root tips was assessed with the CMYB scale using ColorPick v. 3.0 (http://www.iconico.com/colorpic/). The CMYB scale decomposes colours in cyan, magenta, yellow and black components. Hence, the black colour content is annotated ranging from 0, when completely white, to 1, when completely black. Three locations per root tip were selected (as shown in Fig. S6.4) and the content in black annotated by clicking with the mouse. The final colour of a root tip was the average number of the three records in each root tip.

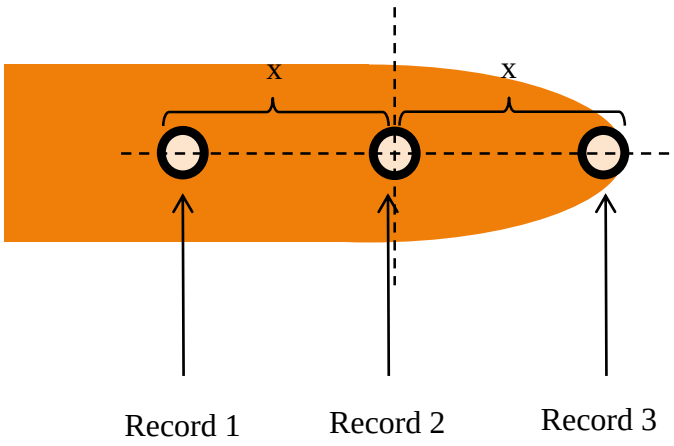


Figure S6.4 Schematic diagram of the location of the three points for colour recording in ECM root tips.

The darkness of the root tips, or the content in black colour, is directly related with the melanin content of fungi, in accordance with classical visual criteria used to differentiate between melanised and non-melanised fungi (Fernández et al., 2016). Chand et al. (2014), for instance, classified fungi as white, mixed and black, and found that the melanin content was related to this classification. We applied our colorimetric approach to the photographs published by Fernandez and Koide (2014) by recording the colour in three random locations of each photograph. We found a good correlation between black colour and melanin contents measured in that publication (Fig. S6.5).

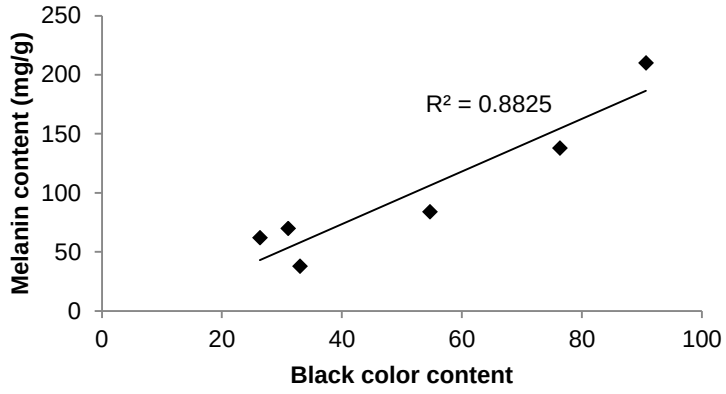


Figure S6.5 Relationship between melanin content and black colour of fungal mycelia. The analysis corresponds to the photographs and melanin contents published by Fernandez and Koide (2014).

Calculation of species trait values

The frequency of emanating hyphae and rhizomorphs of each ectomycorrhizal fungal species was calculated as the proportion of root tips showing those traits in the whole study. Thus:

$$Trait\ vlue = \frac{n_i}{N_i}$$

where n_i is the number of root tips with either emanating hyphae or rhizomorphs of the i-th species and N_i is the total number of root tips belonging to i-th species in the whole study. It resembles the fixed trait value described in Lepš et al. (2011) which is independent from the habitat conditions where the species is found.

Melanisation was calculated as the mean value of black colour content across all root tips belonging to a species. Thus:

$$Melanisation = \frac{\sum_{j=1}^{N_i} black_{ij}}{N_i}$$

where $black_{ij}$ is the colour content of i-th species in j-th root tip and N_i is the total number of root tips belonging to i-th species in the whole study. It is the fixed trait value described by Lepš et al. (2011).

Bibliography

Agerer, R., 2006. Fungal relationships and structural identity of their ectomycorrhizae. Mycological Progress 5, 67-107.

Agerer, R., 2001. Exploration types of ectomycorrhizae: a proposal to classify ectomycorrhizal mycelial systems according to their patterns of differentiation and putative ecological importance. Mycorrhiza 11, 107-114.

Chand, R., Kumar, M., Kushwaha, C., Shah, K., Joshi, A.K. 2014. Role of melanin in release of extracellular enzymes and selection of aggressive isolates of *Bipolaris sorokiniana* in barley. Current Microbiology, 69, 202–211.

Clemente, L., Cabrera, F., García, L.V., Cara, J.S. 2000. Reconocimiento de suelos y estudio de su contaminación por metales pesados en el valle del Guadiamar. Edafología, 7-3, 337-349.

Fernandez, C.W., Koide, R.T. 2014. Initial melanin and nitrogen concentrations control the decomposition of ectomycorrhizal fungal litter. Soil Biology and Biochemistry, 77, 150–157.

Fernandez, C.W., Langley, J.A., Chapman, S., McCormack, M.L., Koide, R.T. 2016. The decomposition of ectomycorrhizal fungal necromass. Soil Biology and Biochemistry, 93, 38–49.

Lepš, J., de Bello, F., Šmilauer, P., Doležal, J. 2011. Community trait response to environment: disentangling species turnover vs intraspecific trait variability effects. Ecography, 34, 856–863.

7. MEDIACIÓN DE HONGOS ECTOMICORRÍCICOS EN LAS INTERACCIONES PLANTA – SUELO

Resumen

Aumenta el consenso de que las comunidades microbianas juegan un importante papel como mediadoras de los procesos ecosistémicos. La ecología basada en los rasgos predice que el impacto de las comunidades microbianas en las funciones ecosistémicas estarán mediadas por la expresión de sus rasgos a nivel de la comunidad. El vínculo entre la respuesta de los rasgos de las comunidades microbianas a las condiciones del medio con el funcionamiento de la planta es aún desconocido en los estudios actuales de ecología microbiana.

En este estudio, se analizaron los rasgos funcionales de las especies de hongos ectomicorrícicos con el objetivo de comprender la importancia del ensamblado de la comunidad en las relaciones suelo-planta. Se han estudiado estas interacciones en las encinas que crecen en un gradiente de exposición a elementos traza tras un vertido minero. En particular, se estudió cómo la composición de hongos ectomicorrícicos y sus rasgos morfológicos a nivel de comunidad median las respuestas de la encina a la contaminación.

La taxonomía y la diversidad funcional de los hongos ectomicorrícicos explicaron una alta proporción de la varianza de los rasgos funcionales de la encina, tanto en raíces como en hojas. Las encinas que estuvieron dominadas por los hongos más abundantes *Hebeloma cavipes* y *Thelephora terrestris* mostraron unos rasgos conservadores en relación al espectro económico de la raíz, mientras que las encinas colonizadas por especies ectomicorrícicas raras o poco abundantes presentaron una estrategia adquisitiva. Las raíces más conservadoras presentaron unos hongos con elevada formación de rizomorfos y baja melanización, lo cual podría estar dirigido por una limitación de recursos. La transferencia del suelo a las raíces de elementos traza fue altamente explicada por la composición de especies ectomicorrícicas, con la mayor transferencia encontrada en las encinas colonizadas por *Hebeloma cavipes*. También el contenido en fósforo en las hojas se relacionó con la composición de especies, con una relación positiva con las raíces colonizadas por *Thelephora terrestris*.

Estos resultados apoyan que existe un potencial de los hongos ectomicorrícicos, tanto por su taxonomía como por sus rasgos, en la mediación del estado de la planta hospedadora. También se apoya que los hongos ectomicorrícicos tienen un efecto en el funcionamiento del ecosistema a través de esta mediación en el espectro económico de la planta.

Abstract

There is an increasing consensus that microbial communities have an important role in mediating ecosystem processes. Trait-based ecology predicts that the impact of the microbial communities on ecosystem functions will be mediated by the expression of their traits at community level. The link between the response of microbial community traits to environmental conditions and its effect on plant functioning is a gap in most current microbial ecology studies.

In this study, we analyzed functional traits of ectomycorrhizal fungal species in order to understand the importance of their community assembly for the soil-plant relationships in holm oak trees (*Quercus ilex* subsp. *ballota*) growing in a gradient of exposure to anthropogenic trace element contamination after a metalliferous tailings spill. Particularly, we addressed how the ectomycorrhizal composition and morphological traits at community level mediate plant response to trace element contamination.

Ectomycorrhizal fungal taxonomy and functional diversity explained a high proportion of variance of tree functional traits, both in roots and leaves. Trees where ectomycorrhizal fungal communities were dominated by the abundant taxa *Hebeloma cavipes* and *Thelephora terrestris* showed a conservative root economics spectrum, while trees colonized by rare taxa presented a resource acquisition strategy. Conservative roots presented ectomycorrhizal functional traits characterized by high rhizomorphs formation and low melanisation which may be driven by resource limitation. Soil-to-root transfer of trace elements was highly explained by the ectomycorrhizal fungal species composition, with the highest transfer found in trees whose roots were colonized by *Hebeloma cavipes*. Leaf P was related to ectomycorrhizal species composition, specifically higher leaf P was related to the root colonization by *Thelephora terrestris*.

These findings support the potential of ectomycorrhizal fungal species and functional traits to mediate plant performance and corroborates the overall effects of ectomycorrhizal fungi on ecosystem functioning through their mediation over the plant economics spectrum.

7.1. Introduction

There is an increasing consensus that microbial communities have an important role in mediating ecosystem processes. In recent years, and thanks to the development of molecular approaches, several studies have focused on the interaction between plants and soil microbial communities to reveal the potential of microbes to drive vegetation diversity and dynamics (Bever, 2003; Erktan et al., 2018; Rutten and Gómez-Aparicio, 2018; van der Heijden et al., 2015; Wardle et al., 2004). As vegetation determines how ecosystems function to a large extent, plant microbiomes indirectly affect the provision of multiple ecosystem services (Friesen et al., 2011; van der Putten et al., 2013). In addition, some studies have highlighted the existence of feedback processes between plants and soil organisms (Bever et al., 2010; Brinkman et al., 2010), suggesting not only the potential of microbes to modify plant communities but also the role of plant communities and their traits at structuring microbial community compositions (Bauman et al., 2016; de Vries et al., 2012; López-García et al., 2017).

Although the effect of plant microbiomes on their hosts has often been studied from a taxonomic point of view (Aponte et al., 2010; de Vries et al., 2012; Kurm et al., 2018), little is known about how soil microbial functional traits are affecting the functioning of plant species. It is debatable whether the features of microbes associated to individual plants (species composition and trait distribution) can be actually defined as plant traits, as they are not heritable features, according to the definition of Garnier et al. (2016). Often, microbial traits in the root microbiome are referred as "biotic root traits" (Bardgett et al., 2014). Recently, some authors have considered the use of traits in the root microbiome as an extension of the plant species phenotype for explaining functional changes in plant communities along environmental gradients (Navarro-Fernández et al., 2016; Jespersen et al. 2019).

The influence of the plant microbiome from a trait-based perspective usually requires assessment of individual species in communities (Díaz et al., 2007), and this has been proven to be very challenging when working with microbes (see Crowther et al., 2014). According to the current thinking on ecological assembly, recording traits at individual species level will allow to differentiate between response and effect traits (Zirbel et al., 2017). The links between the response of microbial community-level traits to

environmental conditions and the effects of these microbial traits changes on plant functioning is an important knowledge gap to be filled in current microbial ecology studies, although the existence of these links have been predicted previously (see Koide et al., 2014).

Mycorrhizal fungi are recognized for their importance for plant foraging of soil resources (Tibbett and Sanders, 2002; van der Heijden et al., 2015), particularly in plant species with relatively thick absorptive roots (Eissenstat et al., 2015; Liu et al., 2015). Coevolution of plant and fungal partners has been recently suggested by Chen et al. (2018), based on their description of a root-fungal functional complementarity in nutrient foraging. However, how mycorrhizal and plant traits are interrelated, for example aligned into the common root economics spectrum framework, and how mycorrhizal traits mediates soil-plant relationships are still open questions that need to be addressed (Weemstra et al., 2016).

This mycorrhiza-root association improves plant health by enhancing resistance to diverse stresses like drought, salinity, heavy metals and pathogens, among others (van der Heijden et al., 2015). Therefore, mycorrhizal mediation on plant performance might be especially important in highly stressful environments, such as trace element (TE) contaminated soils. In these soils, mycorrhizal fungi enhance plant nutrition and stress tolerance; they are also involved in the improvement of soil structure, thus promoting the recovery of the functions in the degraded soil (Firmin et al., 2015; van der Heijden and Scheublin, 2007). Association with mycorrhizal fungi can also play an important role in the transfer of trace elements through the soil-root continuum, an issue of special relevance for the management of trace element contaminated sites. For instance, the phytostabilisation approach is a phytoremediation technology that combines the use of soil amendments and plants to immobilize pollutants into the soil, thus reducing the risks of transfer of these pollutants through the aboveground food web (Mendez and Maier, 2008). A prerequisite to apply this approach to large contaminated areas is that the plants used to remediate the soil can retain trace elements at the rhizosphere level, and do not accumulate them into their aboveground biomass (Bolan et al., 2011). In relation to this, ECM fungi may provide protection against metal toxicity through avoidance (i.e. extracellular precipitation, biosorption to cell walls, reduced uptake) and sequestration (i.e. intracellular chelation, compartmentation into fungi vacuoles) (Hartley et al., 1997a; Jentschke and Godbold, 2000). Therefore, phytoremediation of trace element polluted

soils is enhanced by ECM fungi as they adapt to trace element stress promoting the host growth (Wen et al., 2017).

In this study, we analyzed functional traits in ECM fungal species in order to understand the importance of their community assembly in the soil-plant relationships in holm oak trees (*Quercus ilex* subsp. *ballota*). The experimental site was a trace element contaminated area in which the effect of the abiotic factors (i.e. trace element contamination and soil background variables) on the ECM community composition and their functional traits had been already tested (see chapter 6). Root, leaf and ECM traits were analyzed, as well as the taxonomic structure of ECM communities in the rhizosphere of holm oaks growing on remediated soils exhibiting a gradient of anthropogenic trace element contamination.

We aimed to address how the ECM community composition and morphological traits at community level mediate the response of plants to soil trace element pollution. In particular, we aimed to elucidate the role of ECM community in the plant nutritional status and the transfer of TEs through the soil-root-leaf continuum in a large-scale phytoremediation case study.

We hypothesized that (i) plant traits (i.e. morphological and chemical) of holm oak would change along the trace element gradient; (ii) ECM fungal communities, would partly mediate plant response to trace elements, and thus a significant fraction of the plant nutrient status and transfer of trace elements from soils to leaves will be explained by ECM variables (either species composition or functional traits) (iii) ECM fungal communities lead the intraspecific variation of root functional traits.

7.2. Materials and methods

7.2.1. Study area

The study was conducted at the Guadiamar Green Corridor (SW Spain) were soils severely polluted with several trace elements, mainly As, Cd, Cu, Pb, Tl and Zn (Cabrera et al., 1999). We repeated the sampling design in two sites; the North and the South site. See the general description of the study site in chapter 3. As in chapter 6, four different

areas were sampled: two acidic in the South site, one affected by the mine spill and the other unaffected, and two calcareous in the South site, also affected and unaffected by the mine spill, and the holm oak (*Quercus ilex*) was also the studied species.

7.2.2. Sampling design

The study was conducted in April 2016, sixteen years after the application of soil amendments and the plantation of the former agricultural lands with native trees and shrubs. Holm oak was the target species of the study, given that it was intensively used to afforest the alluvial terraces of the affected area. Four sites were selected along a gradient of soil pollution within the Guadiamar Green Corridor: uncontaminated North (UN), uncontaminated South (US), contaminated North (CN) and contaminated South (CS). A sites location map and a general description of these soils as well as their classification is provided in chapter 6. At each site, ten holm oak trees were randomly selected (n = 40 trees). All these trees were planted at the same time (Autumn 2000) and with similar seed provenance.

For each tree, roots were sampled by carefully tracing from the stems of the tree to the roots belowground in the four cardinal directions. Around 200 g of root material was collected from each direction, i.e. subsamples. Root samples were used to characterize the main root functional traits and the ECM community (see chapter 6 for ECM characterization). Soil samples (0-20 cm depth) were taken with an auger from the four directions under each tree canopy and were pooled to a total of 500 g to make a composite sample per tree. Likewise, leaf samples were taken from the four cardinal directions of the tree canopies to obtain a composite sample of leaves for each tree.

7.2.3. Soil chemical analyses

Soil samples were air-dried and sieved to < 2 mm for chemical analysis. Soil pH, Ca, K, Mg, P, NH_4, NO_3, total C, total N and total trace elements were measured and the methodologies are described in chapter 6. Available concentrations of S and trace elements were extracted from samples (< 60 μm) with a 0.1 M Ca_2Cl solution (Houba et

al., 2000) and analyzed by inductively coupled plasma spectrophotometry (ICP-OES) using a Varian ICP 720-ES (simultaneous ICP-OES with axially viewed plasma).

7.2.4. Soil enzyme activities

The activity of three extracellular enzymes involved in C, N and P cycling (b-glucosidase (BGL), N-acetyl-glucosaminidase (NAG) and acid phosphatase (ACP)) was analyzed colorimetrically by incubation with p-nitrophenyl-linked substrates at 37◦C for 1 h, according to Tabatabai (1982), Parham and Deng (2000)and Tabatabai and Bremner (1969), respectively.

7.2.5. ECM species composition and functional traits

Molecular analysis of ECM in root samples, as well as quantification of ECM functional traits (abundance of rhizomorphs, emanating hyphae and melanin content) were conducted as described in chapter 6. Briefly, a composite sample of 28 root fragments per tree was obtained by selecting the seven longest root fragments in each of the four root subsamples collected from each tree. A random individual root tip per root fragment was photographed for posterior trait quantification (presence of emanating hyphae and rhizomorph and colorimetric estimation of melanisation (see chapter 6). Community weighted means (CWMs), i.e. the averaged value for these traits per tree, was calculated as the number of root tips exhibiting emanating hyphae or rhizomorphs divided by the total number of quantified root tips (Lepš et al., 2011). The color value was averaged between the 28 root tips of each tree for having an overall estimation of the ECM melanisation of the community. The remaining material was used for the quantification of the percentage of root length colonized by ECM fungi, using the gridline intersect method (Brundrett et al., 1996; Navarro-Fernández et al., 2016)

A small portion of each root tip was cut and immersed separately into 10 µl of Extraction Solution (Extract-N-Amp™ Plant PCR Kit by Sigma-Aldrich) and the protocol of the manufacturer was followed to extract its DNA. PCR amplification was conducted using

primers ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990), following the procedure described in chapter 6, and sanger sequenced. Sequences were blasted against the UNITE database (Kõljalg et al., 2013) and those found to correspond to ECM fungi were grouped by genera or family (see chapter 6 for details) and compared against the UNITE database (Kõljalg et al., 2013) for their taxonomic placement and Species Hypothesis determination. The number of root tips belonging to each root was used as abundance data.

7.2.6. Plant functional traits

Root and leaf functional traits were measured following the protocol described in Pérez-Harguindeguy et al. (2013). Morphological root traits included specific root length (SRL), specific root area (SRA) and root dry matter content (RDMC) and were measured with WinRHIZO 2009 (Regent Instruments, Quebec, CA). Specific leaf area (SLA) and leaf dry matter content (LDMC) were measured in a subsample of ten leaves per tree: leaves were scanned and analyzed with Image-Pro 4.5 (Media Cybernetic, Rockville, MD, US).

After sampling we selected ten leaves from each tree and washed them with deionized water to determine the Chlorophyll Content Index (CCI) with a SPAD-502 chlorophyll meter (Minolta Camera Co. Ltd. Osaka, Japan) taking three measurements per leaf.

Subsamples of roots and leaves collected from each tree were used for chemical analysis. These subsamples were washed with distilled water, dried at 70 ºC for at least 48 h, and ground. Total C and N was determined by using a Flash 2000 HT elemental analyzer (Thermo Scientific, Bremen, Germany). Trace elements (As, Cd, Cu, Fe, Mn, Ni, Pb and Zn) and macronutrients (S, P, K, Ca and Mg) were determined by ICP-OES after digestion of plant tissues by wet oxidation with concentrated HNO_3 in a Digiprep MS block digester.

7.2.7. Data analysis

In order to remove correlations and to reduce collinearity between soil variables a principal components analysis (PCA) was used to select a subset of soil trace elements to

be used as predictors of plant traits in subsequent statistical analysis. Original data was log-transformed for normalization. Most correlated trace elements with the first two axes of each PCA were selected for subsequent analyses.

In order to reduce ECM fungal species composition into a less number of dimensions, a principal coordinate analysis (PCoA) was performed with the Operational Taxonomic Units (OTUs) Hellinger transformed abundance data matrix calculated using Euclidean distances (Legendre and Gallagher, 2001). The first two PCoA axes, which explained most of variance in ECM community composition, were selected for further analyses (Table S7.1) (Pinheiro and Bates, 2000; Zuur, 2009).

Differences in soil, root and leaf abiotic and biotic variables among sites were assessed by ANOVA after checking for normality and homoscedasticity. When residuals did not meet the assumption of normality variables were log or square root transformed. When variance heterogeneity was not meet a constant variance function (*varIdent* function in *nlme* R package) was performed. When both assumptions were met a Tukey´s Honest post-hoc test followed. Otherwise, a non-parametric Kruskal Wallis test and a Dunn test corrected by Bonferroni *post-hoc* was performed.

To evaluate the influence of soil and ECM variables on plant nutritional status and functional traits we applied both correlational analysis and linear mixed models between plant and soil/ECM variables (Fig. S7.1). For these analyses, plant variables selected as response variables included root and leaf C, N and P, root morphological traits (SRL, SRA, RDMC), leaf morphological traits (SLA, LDMC, CCI), root-to-soil transfer ratio of trace elements (RS) and root-to leaf transfer ratios of trace elements (i.e. translocation factor (TF)) (Fig. S7.1). Soil factors selected as predictor variables included nutrient concentrations, total and available trace elements and enzyme activities. Finally, ECM fungal factors used as predictors included species composition (i.e. PCoA1 and PCoA2) and functional traits (i.e. emanating hyphae, melanisation and rhizomorphs) (Fig. S7.1).

In order to understand the relationships between these response and predictor variables, we first performed Pearson´s correlation tests, adjusted with Benjamini-Hochberg correction (Benjamini and Hochberg, 1995) to control for ‘‘false discovery rate’’ derived from multiple testing. Those predictors showing a significant relationship with plant response variables were considered as fixed effect factors in a subsequent modelling procedure. Univariate linear mixed effects models, with sampling site as random factor, were run for every selected predictor and every response variable at a time. Only those

showing a significant effect on plant traits, were kept for the subsequent analysis. Finally, multivariate models with the different selected predictors included additively were run for each plant trait as response variable. For these predictors the variance inflated factors (VIF) were calculated and variables with VIF > 3 were removed (Zuur et al., 2010). Models were compared against a null model, assuming no influence of any of these predictors on plant variables. All model combinations (without interaction) were performed and the best and most parsimonious predictive model was selected based on the Akaike information criterion corrected for small sample sizes (AICc; Burnham and Anderson, 2002).

Selected models were fitted and marginal and conditional R^2 values were computed. Marginal R^2 (R^2_{LMMm}) is variance explained by fixed factors, while conditional R^2 (R^2_{LMMc}) is variance explained by both fixed and random factors (Nakagawa and Schielzeth, 2013). Requirements for normality and homoscedasticity of residuals were fulfilled in all the selected models.

All statistical analyses were carried out using the R software v.3.3.2 (R Core Team, 2016) using vegan packages *ggplot2* (Wickham, 2016), *MuMIn* (Barton, 2017), *nlme* (Pinheiro et al., 2016), *psych* (Revelle, 2017) and *vegan* (Oksanen et al., 2016).

7.3. Results

7.3.1. Soil characterisation

Soil pH was significantly different among sites; both sites at the South (US and CS) showed a significantly higher pH than sites in the North (CN and, specially, than UN) (Table S7.1). About soil nutrients, the sites in the North showed significantly higher NH_4, NO_3, total N and organic C than the sites in the South. Calcium concentration was significantly higher at US with respect to the other sites. Phosphorous contents were not significantly different among sites (Table S7.1). All soil enzyme activities presented the highest activity at the CN site and NAG and ACP activities were found significantly lower at US site (Table S7.1).

7.3.2. Reduction of trace element and community composition variables for model analysis

Soil total trace elements PC1 and PC2 ordination axis explained most of the total variance (86.68%) in the chemical composition of soils (Fig. 7.1A). Axis 1 and 2 represented the variation of two clear groups of trace elements which were orthogonal to each other. Axis 1 correlated well with total As, Cd, Cu, Pb, S and Zn, which tended to covariate. Axis 2 showed a high covariance between Mn and Ni. Likewise, the first two axes of available trace elements explained most of the total variance (83.24%) (Fig. 7.1B), being Zn and Mn the most correlated with axes PC1 and PC2 respectively. The final selected trace elements included in the subsequent analyses were: total As, Fe and Mn, and available Mn and Zn concentrations. Available Cd was not chosen because some of the samples were below the detection limits. Lower Guideline Values (LGV) for contaminated soils (Ministry of the Environment Finland, 2007) were exceeded for As, Cu and Pb at UN site (Table S7.1).

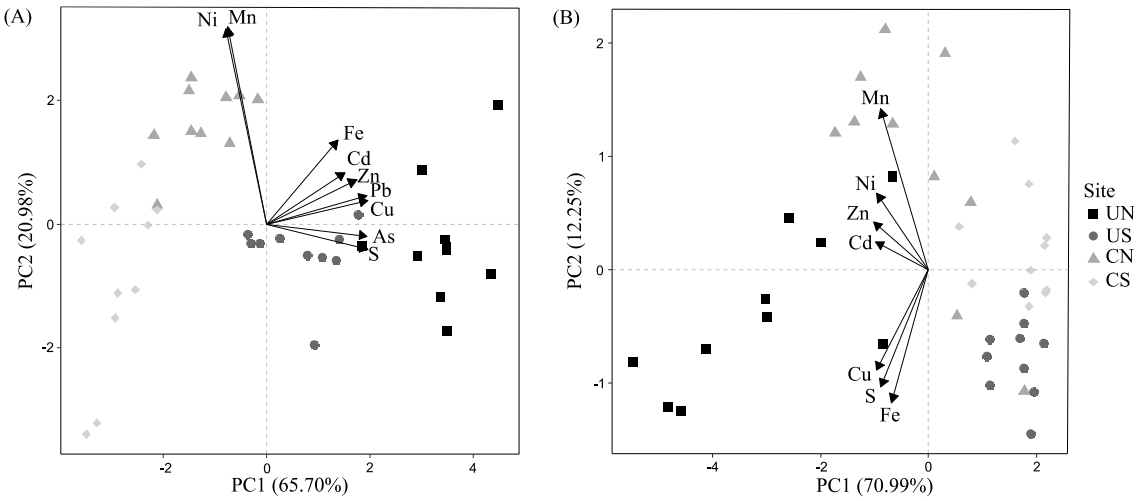


Figure 7.1 Principal component analysis ordination of A) soils total trace elements and B) soil available trace elements, sampled at 0-20 cm depth under holm oak tress (n = 40) and classified by site.

55 OTUs were recorded belonging to ECM fungal species in 494 successfully sequenced root tips. In summary, these taxa comprised 14 families and 19 genera. The presence of rare species was common among the study: 19 of 55 OTUs were only identified in one root tip (Fig. S7.2). Two species, *Hebeloma cavipes* and *Thelephora terrestris* dominated the communities with 83 and 61 root tips, respectively (Fig. S7.2). The first two axes of

PCoA of the ECM fungal communities explained a 25.08% of the variance in community composition. PCoA axis 1 (13.36%) showed a gradient from rare to abundant species (Fig. 7.2). A clear pattern was also found in PCoA axis 2 (11.72% of explained variance) showing a gradient from the presence of *Thelephora terrestris* species to *Hebeloma cavipes* species.

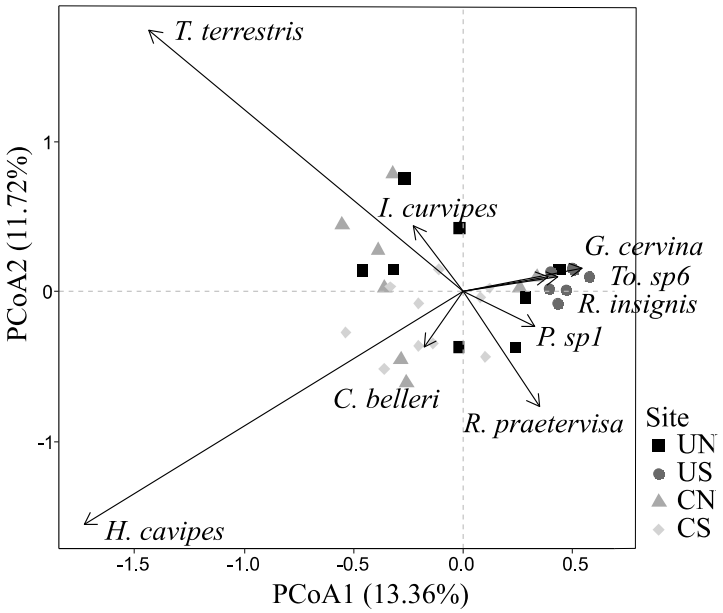


Figure 7.2 Principal coordinate analysis ordination of ectomycorrhizal species in symbiosis with holm oak roots, classified by site. Arrows indicate significant ectomycorrhizal species ($p < 0.05$).

Due to the reduced number of ECM traits and enzyme activities, and the greater variability of the rest of soil background variables (see chapter 6), no initial selection on these sets was carried out.

7.3.3. Relationships between soil and ECM variables and root traits

In general, nutritional root status was found to be more affected by biotic factors than by abiotic ones when univariate models were run. Root C was the variable that was best explained by the considered predictors factor (Table 7.1). Soil Ca and available Mn (Estimate = 0.10) were those variables explaining the greatest variation in root C (univariate models), followed by melanisation (Estimate = -0.23) (Table 7.1). Species

composition PCoA1 (Estimate = -1.13), PCoA2 (Estimate = -1.14), and rhizomorph trait (Estimate = 0.08) also presented an effect on root C but to a lesser extent. Soil Ca content presented a negative effect on C root concentration. ECM species composition as PCoA1 and PCoA2 indicated a higher root C when *Hebeloma cavipes* species was dominant. In terms of biotic CWM traits, a low melanin content and high rhizomorph presence were also affecting root C (univariate models). Root N was significantly best explained by single total Zn, which exerted positive effect (Table 7.1). In terms of biotic effects, species composition PCoA1 had a marginally significance influence (Estimate = 0.05). Root C:N ratio corroborated the role of total Zn on root N, as Zn was found negatively correlated to C:N ratio. The best model for root C:N included species composition PCoA1, exerting a negative effect, which confirmed that the presence of the most abundant species, *Hebeloma cavipes* and *Thelephora terrestris*, negatively influenced root N content. Root P was not explained by any abiotic factor but was positively affected by species composition PCoA1 (Table 7.1 and Fig. 7.3A). As well as root N, root P was found to be lower when *Hebeloma cavipes* and *Thelephora terrestris* species were abundant in roots, therefore the symbioses with other species, here considered as rare due to their lower abundance, improved the nutritional status of holm oak roots in terms of P. Root N:P ratio was not significantly explained by any abiotic or biotic factor.

Both morphological root traits, SRA and RDMC were best explained by species composition PCoA1 than by any abiotic factors, but their effects were opposite (Table 7.1). The presence of the most abundant species, *Hebeloma cavipes* and *Thelephora terrestris* reduced SRA but increased RDMC (Fig. 7.3B), while SRL was not significantly explained by any of the measured soil or ECM fungal factors. In summary, a key effect of species composition was found for those variables related to root nutrition variables. The abundance of *Thelephora terrestris* and, in special, *Hebeloma cavipes* species seemed to be related to the high C, C:N ratio and RDMC values, and low N, P and SRA values in holm oak roots.

Marginal and conditional R^2 for all the response variables, except C:N ratio, were similar. Variance explained by conditional R^2 for the C:N ratio response almost doubled the marginal R^2 (Table 7.1).

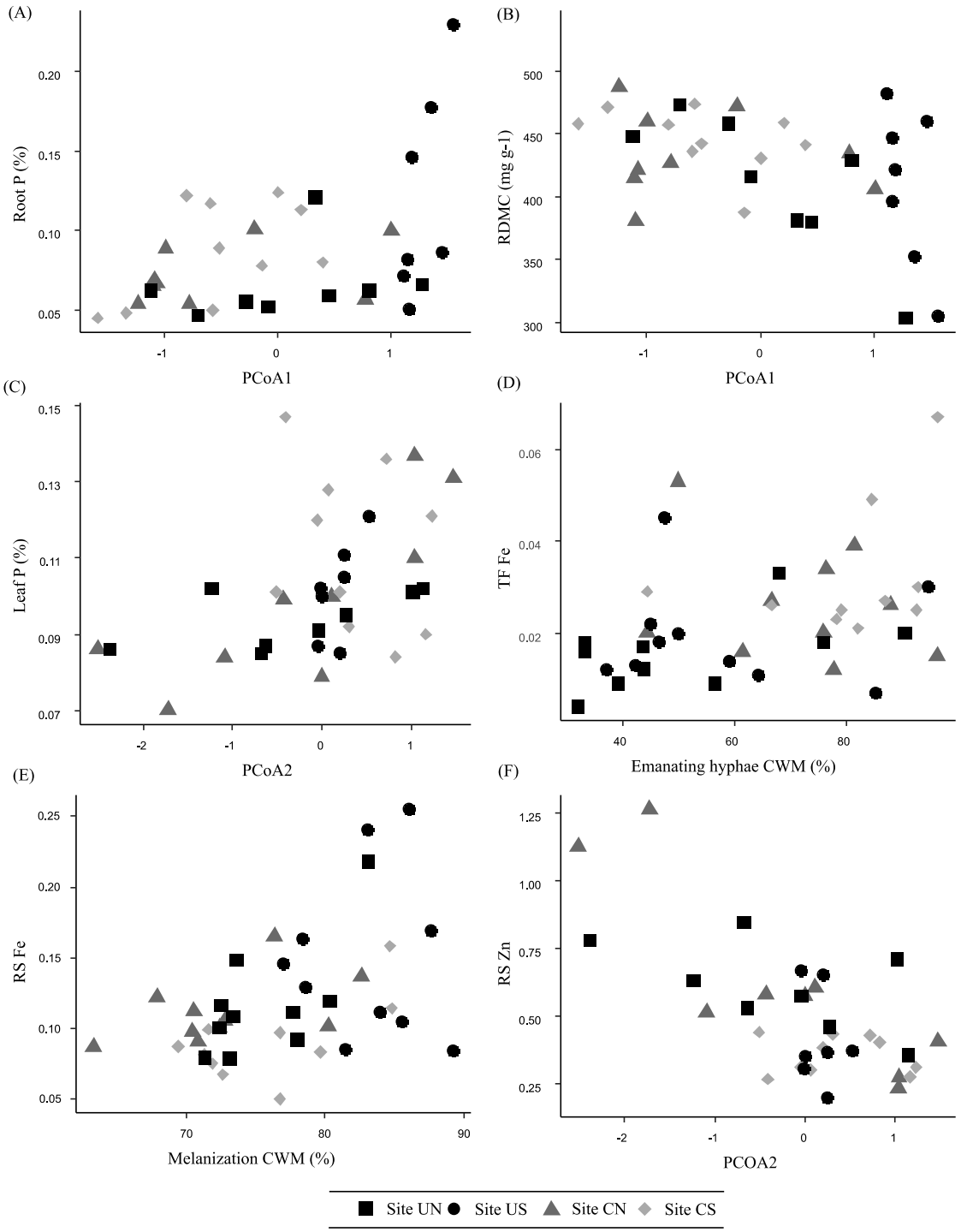


Figure 7.3 Relationships between selected key ectomycorrhizal fungal species composition and traits., and their effects on (A) root P, (B) root dry matter content, (C) leaf P, (D) translocation factor of Fe, (E) soil-to-root Fe transfer, and (F) soil-to-root Zn transfer, classified by sites.

Table 7.1 Univariate and multivariate linear mixed models showing significant soil and ECM fungi fixed effects for each of the root traits and model explained variance. SRA, Specific Root Area; RDMC, Root Dry Matter Content; Av., Available; CWM, Community Weighted Mean; EA, Enzyme Activity; SE, Standard Error; R^2_{LMMm}, marginal variance; R^2_{LMMc}, conditional variance.

Response variable	Individual effects of soil factors				Individual effects of ECM fungal factors				Combining significant effects into the best predictive model					
	Nutrients & EA		Trace Elements		PCoA axis 1 and 2		Fungal traits CWM		Linear mixed effect models				Variance	
Root	Variable	*p*	Variable	*p*	Variable	*p*	Variable	*p*	Model	SE	*t*	*p*	R^2_{LMMm}	R^2_{LMMc}
C (%)	Ca	<0.001	Av. Mn	<0.001	PCoA1	0.048	Melanisation	0.003	C = 45.43 – 0.001 Ca	0.0003	-4.11	<0.001	0.31	0.31
					PCoA2	0.044	Rhizomorph	0.047						
N (%)	-	-	Total Zn	0.006	(PCoA1)	(0.053)	-	-	N = 0.25 + 0.0006 Total Zn	0.0002	2.90	0.006	0.19	0.19
C:N	-	-	Total Zn	0.010	PCoA1	0.012	-	-	C:N = 141.70 – 29.64 PCoA1	11.07	-2.68	0.012	0.22	0.40
P (%)	-	-	-	-	PCoA1	0.009	-	-	P = 0.09 + 0.02 PCoA1	0.007	2.80	0.009	0.21	0.22
SRA (m^2 kg^{-1})	-	-	-	-	PCoA1	0.045	-	-	SRA = 9.47 + 0.62 PCoA1	0.30	2.10	0.045	0.12	0.12
RDMC (mg g^{-1})	-	-	-	-	PCoA1	0.006	-	-	RDMC = 427.04 – 22.04 PCoA1	7.40	-2.98	0.006	0.22	0.22

Table 7.2 Univariate and multivariate linear mixed models showing significant soil and ECM fungi fixed effects for each of the soil-to-root (RS) transfer and translocation factor (TF) and model explained variance. NAG, N-acetyl-glucosaminidase; CWM, Community Weighted Mean; EA, Enzyme Activity; SE, Standard Error; R^2_{LMMm}, marginal variance; R^2_{LMMc}, conditional variance.

Response variable	Individual effects of soil factors				Individual effects of ECM fungal factors				Combining significant effects into the best predictive model					
	Nutrients & EA		Trace Elements		PCoA axis 1 and 2		Fungal traits CWM		Linear mixed effect models				Variance	
Transfer	Variable	*p*	Variable	*p*	Variable	*p*	Variable	*p*	Model	SE	t	*p*	R^2_{LMMm}	R^2_{LMMc}
RS As	-	-	Total As	0.036	PCoA2	0.008	Melanisation	0.050	RS As = -0.32 - 0.04 PCoA2 + 0.006 Melanisation	PCoA2 0.01 Mel 0.002	PCoA2 -3.54 Mel 2.90	PCoA2 0.001 Mel 0.007	0.27	0.63
RS Fe	NO_3	<0.001	-	-	PCoA1	0.002	Melanisation Rhizomorph	0.004 0.021	RS Fe = -0.12 + 0.009 NO_3 + 0.003 Melanisation	NO_3 0.002 Mel 0.0009	NO_3 4.39 Mel 2.99	NO_3 <0.001 Mel 0.005	0.48	0.51
RS Mn	Ca	0.003	Total Mn	0.004	PCoA2	0.039	-	-	RS Mn = 0.61 – 0.00005 Ca - 0.0003 Total Mn	Ca 0.00001 Total Mn 0.00009	Ca -4.09 Total Mn -3.83	Ca <0.001 Total Mn <0.001	0.44	0.44
RS Zn	Ca	<0.001	-	-	PCoA2	<0.001	Melanisation	0.041	RS Zn = 0.50 - 0.16 PCoA2	0.03	-5.29	<0.001	0.46	0.51
TF Fe	-	-	-	-	-	-	Hyphae	0.026	TF Fe = 0.009 + 0.0002 Hyphae	0.0001	2.32	0.026	0.13	0.13
TF Mn	NAG	<0.001	-	-	PCoA1	0.004	-	-	TF Mn = 3.62 + 1.82 PCoA1	0.58	3.15	0.004	0.16	0.75
TF Zn	-	-	Total Zn	0.007	-	-	-	-	TF Zn = 0.98 – 0.002 Total Zn	0.0007	-2.88	0.007	0.22	0.61

Table 7.3 Univariate and multivariate linear mixed models showing significant soil and ECM fungi fixed effects for each of the leaf traits and model explained variance. SLA, Specific Leaf Area; CCI, Chlorophyll Content Index; NAG, N-acetyl-glucosaminidase; Av., Available; CWM, Community Weighted Mean; EA, Enzyme Activity; SE, Standard Error; R^2_{LMMm}, marginal variance; R^2_{LMMc}, conditional variance.

Response variable	Individual effects of soil factors				Individual effects of ECM fungal factors				Combining significant effects into the best predictive model					
	Nutrients & EA		Trace Elements		PCoA axis 1 and 2		Fungal traits CWM		Linear mixed effect models				Variance	
Leaf	Variable	*p*	Variable	*p*	Variable	*p*	Variable	*p*	Model	SE	t	*p*	R^2_{LMMm}	R^2_{LMMc}
C (%)	-	-	Total As	0.014	PCoA1	0.010	Hyphae	<0.001	C= 48.56 + 1.93 PCoA1 + 0.019 Total As	PCoA1 0.50 Total As 0.006	PCoA1 3.86 Total As 2.87	PCoA1 <0.001 Total As 0.008	0.51	0.51
N (%)	P	<0.001	-	-	-	-	-	-	N = 1.20 + 0.001 P	0.002	4.45	<0.001	0.35	0.35
C:N	P	<0.001	-	-	PCoA1	0.048	-	-	C:N = 42.10 – 0.32 P	0.09	-3.47	<0.001	0.25	0.26
P (%)	P	0.035	Av. Mn	0.007	PCoA2	0.007	-	-	P = 0.10 + 0.009 PCoA2	0.003	2.91	0.007	0.21	0.21
N:P	-	-	-	-	PCoA2	0.039	-	-	N:P = 13.30 – 0.90 PCoA2	0.42	-2.16	0.039	0.13	0.13
SLA (m^2 kg^{-1})	NAG	0.002	-	-	-	-	-	-	SLA = 4.38 + 1.10 NAG	0.33	3.35	0.002	0.24	0.24
CCI (SPAD)	P	<0.001	-	-	-	-	-	-	CCI = 44.37 + 0.30 P	0.07	4.38	<0.001	0.30	0.49

7.3.4. Relationships between soil and ECM variables and transfer of trace elements to roots

Transfer of trace elements from soil to root seemed to be mainly driven by biotic factors: species composition PCoA2 and melanisation CWM (Table 7.2). The soil-to-root transfer of As (RS As) was related to species composition PCoA2 and ECM melanisation (Table 7.2). A high abundance of *Hebeloma cavipes* species and a high melanin content seemed to be associated to a high As transfer. A negative relationship with soil As was also found (Estimate = -0.0004). The soil-to-root transfer of Fe (RS Fe) was positively explained by soil NO_3 and melanisation (Table 7.2 and Fig. 7.3E). Species composition PCoA1 was positive related (Estimate = 0.026) meaning that in those soils where rare species were abundant, Fe transfer was higher. Rhizomorphs formation was negatively related (Estimate = -0.01). The soil-to-root transfer of Mn (RS Mn) was best explained by abiotic variables, namely soil Ca and soil Mn, which negatively associated with this transfer (Table 7.2). Species composition PCoA2 showed an individual negative effect on Mn transfer (Estimate = -0.06), therefore the abundance of *Hebeloma cavipes* species in the soil was found to be positively related to Mn transfer from soils to roots. The soil-to-root transfer of Zn (RS Zn) was negatively affected by species composition PCoA2 (Table 7.2 and Fig. 7.3f). Therefore, as previously found in As, a higher abundance of *Hebeloma cavipes* species increased the soil-to-root transfer of Zn. In this case, the significant effect of melanisation was negative (Estimate = -0.01), opposite to As and Fe transfers. Soil Ca (Estimate = -0.00009) showed an individual negative effect on soil-to-root transfer of Zn.

Marginal and conditional R^2 showed similar percentage of variances for Fe, Mn and Zn transfer but transfer of As was more explained by the site random effect (conditional R^2) than the biotic fixed effects (Table 7.2).

7.3.5. Relationships between soil and translocation of trace elements to leaves

Translocation of trace elements from roots to leaves were explained by different abiotic and biotic factors (Table 7.2), depending on the element. Translocation of As was not significantly explained by any individual abiotic or biotic factor. The closest variable

showing an effect on As transfer was species composition PCoA2 (t = 1.67; p = 0.105), although most of the variability in this model was explained by the site random effect (R^2_{LMMc}= 0.52). Due to the non-significant fixed effect of the model for the response variable translocation factor of As, a covariate Cu transfer was studied. Translocation factor of Cu was highly explained by soil Cu and species composition PCoA2 (R^2_{LMMm}= 0.55; R^2_{LMMc}= 0.76). Soil Cu contamination showed a significant negative effect on the Cu translocation (p <0.001) while PCoA2 showed a significant positive effect (p = 0.013), therefore Cu translocation was favored on *Thelephora terrestris* dominated soils. Iron translocation from roots to leaves was only significantly explained by the biotic emanating hyphae, showing a positive relationship (Table 7.2 and Fig. 7.3D). Translocation factor of Mn was significantly related to NAG enzyme activity and species composition PCoA1, being this last variable the most explicative, showing a positive effect (Estimate = 8.54) (Table 7.2). Translocation factor of Zn was only significantly explained by soil Zn, however Zn showed a negative effect on Zn transfer (Table 7.2).

High differences between marginal and conditional R^2 variance were found for all trace elements translocation factors, except for Fe (Table 7.2).

7.3.6. Relationships between soil and leaf traits

Nutritional status of holm oak leaves were, in general, highly affected by soil P and biotic species composition (PCoA1 and PCoA2 factors; Table 7.3). Leaf C was highly explained by a combination of abiotic and biotic factors (soil As and species composition PCoA1 factors, Table 7.3). Both predictor variables showed a strong positive relationship with leaf C. Emanating hyphae was also found to influence leaf C content, but negatively (Estimate = -0.09), when univariate relationships were analyzed. Leaf N was significantly influenced by soil P (Table 7.3) which explained a high proportion of variance of leaf N. No biotic effect was identified as significant for leaf N. Leaf C:N ratio was also highly explained by soil P but a negative effect was registered, in coherence with leaf N effects. A biotic effect was significantly found in relation to species composition PCoA1. The positive effect (Estimate = 2.04) of PCoA1 on this ratio showed consistency with model effects on leaf C. In summary, the results from these models showed that a higher leaf C content and, therefore a higher C:N ratio, in those sites with particular abiotic

characteristics (high As contamination and low soil P), and with certain biotic features: low abundance of *Hebeloma cavipes* and *Thelephora terrestris*, and low emanating hyphae.

Leaf P was best explained by species composition PCoA2 alone, which had a positive effect on this response variable (Table 7.3 and Fig. 7.3C). Soil P and available Mn had also a significant influence on leaf P, according to abiotic univariate models; soil P had a positive effect (Estimate = 0.0007) while Mn availability showed a negative effect (Estimate = -0.0005) on leaf P. Leaf N:P ratio was best predicted by species composition PCoA2 alone; the negative effect between PCoA2 and this the ratio corroborates the previous leaf P results. No abiotic variables were found to have a significant effect on leaf N:P ratio. To summarize, a higher leaf P and a lower N:P ratio were found in soils with high P, low Mn availability and dominance of *Thelephora terrestris* over *Hebeloma cavipes* species.

Morphological trait SLA was best explained by soil NAG enzyme activity, while CCI was significantly related to soil P. For both SLA and CCI no mycorrhizal variables were predictors of their variance (Table 7.3). No significant variables were found to explain LDMC variation.

Marginal and conditional R^2 for all the leaf response variables were akin except for CCI which presented a higher conditional variance (Table 7.3).

7.4. Discussion

In this study we aimed to quantify the influence of ECM fungal communities on certain plant morphological and chemical traits, and to assess whether this influence can overcome that of the abiotic environment. The scenario chosen for this purpose was a trace element contaminated area in which the effect of the abiotic factors, including the trace element contamination and the soil background variables, on the community composition and functional traits of ECM fungi had been already tested in chapter 6. Hence, since the abiotic environment was indeed shaping the ECM communities, any effect of the latter on plant traits must be interpreted as a mediated effect of the ECM fungi on soil-plant relationships. In general, we found that ECM community composition

and traits explained more than the abiotic environment for most of the measured plant traits.

7.4.1. Root functional traits

Root systems are known to show a high plasticity in their development depending on soil local heterogeneity (Ostonen et al., 2007). In this study, we found several significant relationships between soil variables and root traits in holm oak trees with the same age and origin, which suggests a high root plasticity in response to the studied environmental gradient. We further found that root functional traits were highly explained by the ECM community (in terms of both fungal species and traits), which corroborates the important mediation role of ECM on plant status and performance, and the need of incorporating symbiotic traits into the analysis of root traits (Weemstra et al., 2016).

In relation to the root economics spectrum, we could align the presence of abundant species of ECM (*H. cavipes* and *T. terrestris*) with conservative positions into the root economics spectrum, i.e. exhibiting conservative traits such as a high C:N ratio and a low N and P content, and consequently a high C content, high RDMC and low RDA (de la Riva et al., 2018, 2016). The basidiomycete *Thelephora terrestris* is a common symbiotic ECM fungus (Marx et al., 1984; Menkis and Vasaitis, 2011) with beneficial effects for trees growing under stressful conditions, such as those that prevail in mine areas and reclamation sites (Lee and Koo, 1983), given that it protects the host by decreasing metal (Cu) transfer from soil to roots (Van Tichelen et al., 2001). Although *Hebeloma spp.* have been frequently found in heavy-metal contaminated soils (Colpaert et al., 2011) the abundant *Hebeloma cavipes* taxa is associated in the study area with soils with a low level of trace element contamination. In terms of ECM traits, a high rhizomorph formation and a low melanin content characterized those ECM fungi (i.e. *Thelephora terrestris* and *Hebeloma cavipes*) that were colonizing roots showing the most conservative traits. The presence of rhizomorphs, which functionally increases water and phosphate uptake through a long-distance exploration mechanism (Agerer, 2001), may be a consequence of resource limitations, hence constituting a conservative trait. Melanin plays a role in protecting the root cells against high concentrations of heavy metals in the soil (Gadd and de Rome, 1988). Thus, a high level of melanisation might be not essential in these soils with low trace element contamination.

In the opposite edge of the root economics spectrum, we found roots colonized mostly by rare species and showing more acquisitive features, i.e. a high N and P concentration, a low RDMC and high SRA (de la Riva et al., 2018, 2016). These root traits might be indicating less resource limitations, probably due to higher soil nutrient contents and thus less dependency on rhizomorphs for nutrient acquisition. The fact that these roots belong to trees growing in soils with a high level of trace element contamination could explain the higher degree of melanisation of these fungi, in order to avoid trace element toxicity.

In this study, we could have expected that the adverse soil chemical conditions posed by the contamination episode could have modulated root acquisition strategies, with roots growing in the most contaminated soils showing a more conservative strategy. However, conservative root traits were related to low soil trace elements (Zn) concentrations. On one hand, it is possible that the a priori concern about trace elements contamination as the main factor of stress for plant performance is masked by other sources of stress, such as water or nutrient limitations. Recently, in chapter 6 we found that soil background properties and trace elements concentrations explained the same proportion of variance in ECM species composition, which support this concept. On the other hand, although the root economics spectrum is associated to nutrient absorption, here we found that other factors such as ECM community composition and trace element contamination could support the multidimensional root trait framework. Mycorrhizal fungi have a fundamental role in acquiring resources but also plant protection from the negative impact of some sources of biotic and abiotic stress. trace element contamination seems to be independent from root economics spectrum, which indicate the existence of a multidimensional framework that includes other processes different from those related to nutrient uptake (Weemstra et al., 2016).

7.4.2. Soil-root-leaf transfer of trace elements

Trace element mobility through the soil-root-leaf continuum depends on several factors, and obviously initial concentrations in the soil is one of them (Kabata-Pendias, 2004). Despite that the range of soil trace elements concentrations in our environmental gradient was relatively large (for example, total As concentrations ranged from 6.83 to 286 mg kg^{-1}), accumulation of trace elements in oak leaves was relatively low, and leaf trace

elements concentrations were within the normal ranges and below the levels that can be toxic to plants, except Mn levels (over 400 mg kg^{-1}) (Madejón et al., 2002). This confirms that holm oak is a suitable species for the phytostabilization of contaminated soils, given its ability to prevent trace elements accumulation into aboveground biomass (Table S7.1). Previous work under controlled greenhouse conditions showed that this species has a capacity to retain and tolerate high concentrations of some trace elements (Cd) into fine roots (Domínguez et al., 2009). Mechanisms involved in trace elements retention into the root system include adsorption onto roots, or precipitation within the rhizosphere (Pulford and Watson, 2003; Wong, 2003). The pectin in the cell wall are the main constituents allowing metal binding due to their carboxyl groups, which have a high cation exchange capacity (Franco et al., 2002). In the present study, the soil-to-root transfer of As, Mn and Zn was highly explained by ECM fungal species composition and traits, which suggests that interactions with fungi play an important role at determining the capacity of this species to retain trace element into its roots. The highest transfer of these trace elements from soil to roots was observed in trees whose roots were colonized by *H. cavipes*. In contrast, soil-to-root transfer of Fe presented a different trend, with the highest transfer being recorded in roots colonized by rare species taxa. This confirms that the mechanisms by which mycorrhizal fungi participate in metal uptake by plants can be very species-specific (Godbold et al., 1998; Jentschke and Godbold, 2000)

Melanisation was corroborated as a trait with a role in the protection of plants against heavy metals (Gadd and de Rome, 1988), as it was highly related to trace element transfer, although the relationship between melanin content and transfer of trace element from soils to roots was differed across elements. Melanisation was positively related to As and Mn but negatively related to Zn transfer. These opposite trends could indicate that roots are subjected to multiple constraints (Weemstra et al., 2016) in these multi-metal contaminated soils, and that different elements affect differently to these ECM traits.

7.4.3. Leaf functional traits

Resource availability directly impacts functional traits such as SLA and leaf N and P content (Friesen et al., 2011). It was expected that ECM fungal mediation would increase

resource acquisition by plants by accessing to organic forms unavailable for plants and by more efficient foraging (Friesen et al., 2011).

Leaf C is captured via photosynthesis, therefore C uptake is not mediated by ECM fungi. But assimilation of C into plant tissue might be affected by a range of factors, such as nutritional status and water stress, in which ECM community may play certain role, as explored here (Cornelissen et al., 2001). A high positive relationship was found between ECM species composition (mainly, in relation to the presence of rare species species) and leaf C and C:N ratio. Therefore, there might be an indirect effect of ECM community composition through its effects on root functional traits. Leaf P and N:P ratio were related to ECM species composition as well, specifically high leaf P was related to the root colonization by *T. terrestris.* This is in agreement with Van Tichelen et al. (2001), who showed that *T. terrestris* played a central role in the P nutrition of the host plant in a P-limited and Cu-contaminated soil.

Plants are performing a continuous carbon and nutrient investment in order to maintain the key leaf functions (i.e. photosynthesis) (Poorter and Bongers, 2006). Leaf N is responsible of the photosynthetic machinery, especially Rubisco, and leaf P is found in nucleic acids, lipid membranes and bioenergetic molecules (Wright et al., 2004), therefore both are key chemical traits. Leaf N correlated positively with CCI and this result agreed with that an optimal leaf N is essential for photosynthesis (de la Riva et al., 2016). Leaf N, P and CCI have shown a high positive relationship to soil P. A P limitation in soils has been previously registered in the study area (Domínguez et al., 2010b) and it is known that in soils limited by P these traits are more closely linked to this element (Chen et al., 2011; Liu et al., 2010; Niinemets and Kull, 2003).

SLA and CCI were not related to ECM fungal species composition or traits. These functional traits are related to light capturing functions (Niinemets and Sack, 2006) which here have been found not to be mediated by ECM, but affected by soil variables (i.e. NAG and P).

7.5. Conclusion

The analysis of root and leaf traits, as well as ECM communities and soil physico-chemical properties in a large-scale phytoremediated area, revealed that plant functions,

expressed as variations in plant traits, can be affected in similar extents by the abiotic and biotic environment that surround and interact with each individual plant. We could identify some ECM fungal community traits that were highly related to the studied plant variables (root traits, nutrient status and trace element accumulation), in a greater extent than the abiotic environment. In some cases, such as the transfer of As, Mn and Zn, the best explanatory variable was directly related to the composition of the ECM community, suggesting species-specific mechanisms of interactions between holm oak and ECM fungi. ECM traits co-varied with the root economics spectrum, as ECM rhizomorphs and melanisation traits were related to the acquisitive-conservative root spectrum. Future studies on plant-soil interactions in contaminated soils should therefore consider that critical processes, such as nutrient assimilation and trace element accumulation into biomass, can be largely mediated by ECM fungi.

7.6. Bibliography

Agerer, R., 2001. Exploration types of ectomycorrhizae: a proposal to classify ectomycorrhizal mycelial systems according to their patterns of differentiation and putative ecological importance. Mycorrhiza 11, 107–114.

Aponte, C., García, L. V., Marañón, T., Gardes, M., 2010. Indirect host effect on ectomycorrhizal fungi: Leaf fall and litter quality explain changes in fungal communities on the roots of co-occurring Mediterranean oaks. Soil Biology and Biochemistry 42, 788–796.

Bardgett, R.D., Mommer, L., De Vries, F.T., 2014. Going underground: Root traits as drivers of ecosystem processes. Trends in Ecology and Evolution 29, 692–699.

Barton, K., 2017. MuMIn: Multi-Model Inference.

Bauman, D., Raspé, O., Meerts, P., Degreef, J., Muledi, J.I., Drouet, T., 2016. Multiscale assemblage of an ectomycorrhizal fungal community: the influence of host functional traits and soil properties in a 10-ha miombo forest. FEMS Microbiology Ecology 92, 1–14.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate : a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society. Series B (Methodological) 57, 289–300.

Bever, J.D., 2003. Soil community feedback and the coexistence of competitors: conceptual frameworks and empirical tests. New Phytologist 157, 465–473.

Bever, J.D., Dickie, I.A., Facelli, E., Facelli, J.M., Klironomos, J., Moora, M., Rillig, M.C., Stock, W.D., Tibbett, M., Zobel, M., 2010. Rooting theories of plant community ecology in microbial interactions. Trends in Ecology and Evolution 25, 468–478.

Bolan, N.S., Park, J.H., Robinson, B., Naidu, R., Huh, K.Y., 2011. Phytostabilization: a green approach to contaminant containment. Advances in Agronomy 112, 145–204.

Brinkman, E.P., Van der Putten, W.H., Bakker, E.J., Verhoeven, K.J.F., 2010. Plant-soil feedback: Experimental approaches, statistical analyses and ecological interpretations. Journal of Ecology 98, 1063–1073.

Brundrett, M., Bougher, N., Dell, B., Grove, T., Malajczuk, N., 1996. Examining mycorrhizal associations, in: Working with Mycorrhizas in Forestry and Agriculture. pp. 173–193.

Burnham, K.P., Anderson, D.R., 2002. Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach, Second Ed. ed, Ecological Modelling. Springer, New York.

Cabrera, F., Clemente, L., Díaz Barrientos, E., López, R., Murillo, J.M., 1999. Heavy metal pollution of soils affected by the Guadiamar toxic flood. Science of the Total Environment 242, 117–129.

Chen, F.-S., Niklas, K.J., Zeng, D.-H., 2011. Important foliar traits depend on species-grouping: analysis of a remnant temperate forest at the Keerqin Sandy Lands, China. Plant and Soil 340, 337–345.

Chen, W., Koide, R.T., Eissenstat, D.M., 2018. Nutrient foraging by mycorrhizas: from species functional traits to ecosystem processes. Functional Ecology 32, 858–869.

Colpaert, J. V., Wevers, J.H.L., Krznaric, E., Adriaensen, K., 2011. How metal-tolerant ecotypes of ectomycorrhizal fungi protect plants from heavy metal pollution. Annals of Forest Science 68, 17–24.

Cornelissen, J., Aerts, R., Cerabolini, B., Werger, M., Van der Heijden, M., 2001. Carbon cycling traits of plant species are linked with mycorrhizal strategy. Oecologia 129, 611–619.

Crowther, T.W., Maynard, D.S., Crowther, T.R., Peccia, J., Smith, J.R., Bradford, M.A., 2014. Untangling the fungal niche: The trait-based approach. Frontiers in Microbiology 5, 1–12.

de la Riva, E.G., Marañón, T., Pérez-Ramos, I.M., Navarro-Fernández, C.M., Olmo, M., Villar, R., 2018. Root traits across environmental gradients in Mediterranean woody communities: are they aligned along the root economics spectrum? Plant and Soil 1–14.

de la Riva, E.G., Tosto, A., Pérez-Ramos, I.M., Navarro-Fernández, C.M., Olmo, M., Anten, N.P.R., Marañón, T., Villar, R., 2016. A plant economics spectrum in Mediterranean forests along environmental gradients: Is there coordination among leaf, stem and root traits? Journal of Vegetation Science 27, 187–199.

de Vries, F.T., Manning, P., Tallowin, J.R.B., Mortimer, S.R., Pilgrim, E.S., Harrison, K.A., Hobbs, P.J., Quirk, H., Shipley, B., Cornelissen, J.H.C., Kattge, J., Bardgett, R.D., 2012. Abiotic drivers and plant traits explain landscape-scale patterns in soil microbial communities. Ecology Letters 15, 1230–1239.

Díaz, S., Lavorel, S., de Bello, F., Quétier, F., Grigulis, K., Robson, T.M., 2007. Incorporating plant functional diversity effects in ecosystem service assessments. Proceedings of the National Academy of Sciences of the United States of America 104, 20684–20689.

Domínguez, M.T., Alegre, J.M., Madejón, P., Madejón, E., Burgos, P., Cabrera, F., Marañón, T., Murillo, J.M., 2016. River banks and channels as hotspots of soil pollution after large-scale remediation of a river basin. Geoderma 261, 133–140.

Domínguez, M.T., Madrid, F., Marañón, T., Murillo, J.M., 2009. Cadmium availability in soil and retention in oak roots: potential for phytostabilization. Chemosphere 76, 480–486.

Domínguez, M.T., Marañón, T., Murillo, J.M., Schulin, R., Robinson, B.H., 2010. Nutritional status of Mediterranean trees growing in a contaminated and remediated area. Water, Air, and Soil Pollution 205, 305–321.

Domínguez, M.T., Marañón, T., Murillo, J.M., Schulin, R., Robinson, B.H., 2008. Trace element accumulation in woody plants of the Guadiamar Valley, SW Spain: a large-scale phytomanagement case study. Environmental Pollution 152, 50–59.

Eissenstat, D.M., Kucharski, J.M., Zadworny, M., Adams, T.S., Koide, R.T., 2015. Linking root traits to nutrient foraging in arbuscular mycorrhizal trees in a temperate forest. New Phytologist 208, 114–124.

Erktan, A., McCormack, M.L., Roumet, C., 2018. Frontiers in root ecology: recent advances and future challenges. Plant and Soil 424, 1–9.

Firmin, S., Labidi, S., Fontaine, J., Laruelle, F., Tisserant, B., Nsanganwimana, F., Pourrut, B., Dalpé, Y., Grandmougin, A., Douay, F., Shirali, P., Verdin, A., Lounès-Hadj Sahraoui, A., 2015. Arbuscular mycorrhizal fungal inoculation protects Miscanthus × giganteus against trace element toxicity in a highly metal-contaminated site. Science of the Total Environment 527–528, 91–99.

Franco, C.R., Chagas, A.P., Jorge, R.A., 2002. Ion-exchange equilibria with aluminum pectinates. Colloids and Surfaces A: Physicochemical and Engineering Aspects 204, 183–192.

Friesen, M.L., Porter, S.S., Stark, S.C., von Wettberg, E.J., Sachs, J.L., Martinez-Romero, E., 2011. Microbially Mediated Plant Functional Traits. Annual Review of Ecology, Evolution, and Systematics 42, 23–46.

Gadd, G.M., de Rome, L., 1988. Biosorption of copper by fungal melanin. Applied Microbiology and Biotechnology 29, 610–617.

Gardes, M., Bruns, T.. D., 1993. ITS primers with enhanced specificity for basidiomycetes—Application to identification of mycorhizae and rusts. Molecular Ecology 2, 113–8.

Garnier, E., Navas, M.-L., Grigulis, K., 2016. Plant functional diversity : organism traits, community structure, and ecosystem properties. Oxford University Press, Oxford.

Godbold, D.L., Jentschke, G., Winter, S., Marschner, P., 1998. Ectomycorrhizas and amelioration of metal stress in forest trees. Chemosphere 36, 757–762.

Hartley, J., Cairney, J.W.G., Meharg, A.A., 1997. Do ectomycorrhizal fungi exhibit adaptive tolerance to potentially toxic metals in the environment? Plant and Soil 189, 303–319.

Houba, V.J.G., Temminghoff, E.J.M., Gaikhorst, G.A., van Vark, W., 2000. Soil analysis procedures using 0.01 M calcium chloride as extraction reagent. Communications in Soil Science and Plant Analysis 31, 1299–1396.

Jentschke, G., Godbold, D.L., 2000. Metal toxicity and ectomycorrhizas. Physiologia Plantarum 109, 107–116.

Jespersen J.R.P., Liengaard Johansen J., Rabelo Pereira, C.M., Bruun H.H, Rosendahl S., Kjøller R., Lopez-García A., 2019. Mycorrhizal features and leaf traits covary at the community level during primary succession. Fungal Ecology 40, 4-11.

Kabata-Pendias, A., 2004. Soil-plant transfer of trace elements - An environmental issue. Geoderma 122, 143–149.

Koide, R.T., Fernandez, C., Malcolm, G., 2014. Determining place and process: functional traits of ectomycorrhizal fungi that affect both community structure and ecosystem function. New Phytologist 201, 433–439.

Kõljalg, U., Nilsson, R.H., Abarenkov, K., Tedersoo, L., Taylor, A.F.S., Bahram, M., Bates, S.T., 2013. Towards a unified paradigm for sequence-based identification of fungi. Molecular Ecology 22, 5271–5277.

Kurm, V., Van Der Putten, W.H., Pineda, A., Hol, W.H.G., 2018. Soil microbial species loss affects plant biomass and survival of an introduced bacterial strain, but not inducible plant defences. Annals of Botany 121, 311–319.

Lee, K.J., Koo, C.D., 1983. Inoculation of pines in a nursery with Pisolithus tinctorius and Thelephora terrestris in Korea, in: Tree Root Systems and Their Mycorrhizas. Springer Netherlands, Dordrecht, pp. 325–329.

Legendre, P., Gallagher, E.D., 2001. Ecologically meaningful transformations for ordination of species data. Oecologia 129, 271–280.

Lepš, J., de Bello, F., Šmilauer, P., Doležal, J., 2011. Community trait response to environment: Disentangling species turnover vs intraspecific trait variability effects. Ecography 34, 856–863.

Liu, B., Li, H., Zhu, B., Koide, R.T., Eissenstat, D.M., Guo, D., 2015. Complementarity in nutrient foraging strategies of absorptive fine roots and arbuscular mycorrhizal fungi across 14 coexisting subtropical tree species. New Phytologist 208, 125–136.

Liu, G., Freschet, G.T., Pan, X., Cornelissen, J.H.C., Li, Y., Liu, G., Freschet, G.T., Pan, X., Cornelissen, J.H.C., Li, Y., Dong, M., 2010. Coordinated variation in leaf and root traits across multiple spatial scales in Chinese semi-arid and arid ecosystems. New Phytologist 188, 543–553.

López-García, Á., Varela-Cervero, S., Vasar, M., Öpik, M., Barea, J.M., Azcón-Aguilar, C., 2017. Plant traits determine the phylogenetic structure of arbuscular mycorrhizal fungal communities. Molecular Ecology 26, 6948–6959.

Madejón, P., Domínguez, M.T., Madejón, E., Cabrera, F., Marañón, T., Murillo, J.M., 2018. Soil-plant relationships and contamination by trace elements: a review of twenty years of experimentation and monitoring after the Aznalcóllar (SW Spain) mine accident. Science of the Total Environment 625, 50–63.

Madejón, P., Murillo, J.M., Maraón, T., Cabrera, F., López, R., 2002. Bioaccumulation of As, Cd, Cu, Fe and Pb in wild grasses affected by the Aznalcóllar mine spill (SW Spain). Science of the Total Environment 290, 105–120.

Marx, D.H., Cordell, C.E., Kenney, D.S., Mexal, J.G., Artman, J.D., Riffle, J.W., Molina, R.J., 1984. Commercial Vegetative Inoculum of Pisolithus tinctorius and Inoculation Techniques for Development of Ectomycorrhizae on Bare-root Tree Seedlings. Forest Science 30, a0001-z0001.

Mendez, M.O., Maier, R.M., 2008. Phytostabilization of mine tailings in arid and semiarid environments - An emerging remediation technology. Environmental Health Perspectives 116, 278–283.

Menkis, A., Vasaitis, R., 2011. Fungi in Roots of Nursery Grown Pinus sylvestris: Ectomycorrhizal Colonisation, Genetic Diversity and Spatial Distribution. Microbial Ecology 61, 52–63.

Ministry of the Environment Finland, 2007. Government Decree on the Assessment of Soil Contamination and Remediation Needs (214/2007).

Nakagawa, S., Schielzeth, H., 2013. A general and simple method for obtaining R2 from generalized linear mixed-effects models. Methods in Ecology and Evolution 4, 133–142.

Navarro-Fernández, C.M., Pérez-Ramos, I.M., de la Riva, E.G., Vera, J.R., Roumet, C., Villar, R., Marañón, T., 2016. Functional responses of Mediterranean plant communities to soil resource heterogeneity: a mycorrhizal trait-based approach. Journal of Vegetation Science 27, 1243–1253.

Niinemets, Ü., Kull, K., 2003. Leaf structure vs. nutrient relationships vary with soil conditions in temperate shrubs and trees. Acta Oecologica 24, 209–219.

Niinemets, Ü., Sack, L., 2006. Structural determinants of leaf light-harvesting capacity and photosynthetic potentials. Progress in Botany 67, 385–419.

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., 2016. vegan: Community Ecology Package.

Ostonen, I., Püttsepp, Ü., Biel, C., Alberton, O., Bakker, M.R., Lõhmus, K., Majdi, H., Metcalfe, D., Olsthoorn, A.F.M., Pronk, A., Vanguelova, E., Weih, M., Brunner, I., 2007. Specific root length as an indicator of environmental change. Plant Biosystems 141, 426–442.

Parham, J.A., Deng, S.P., 2000. Detection, quantification and characterization of b - glucosaminidase activity in soil. Soil Biology & Biochemistry 32, 1183–1190.

Pérez-Harguindeguy, N., Díaz, S., Garnier, E., Lavorel, S., Poorter, H., Jaureguiberry, P., Bret-Harte, M.S., Cornwell, W.K., Craine, J.M., Gurvich, D.E., Urcelay, C., Veneklaas, E.J., Reich, P.B., Poorter, L., Wright, I.J., Ray, P., Enrico, L., Pausas, J.G., De Vos, A.C., Buchmann, N., Funes, G., Quétier, F., Hodgson, J.G., Thompson, K., Morgan, H.D., Ter Steege, H., Van Der Heijden, M.G.A., Sack, L., Blonder, B., Poschlod, P., Vaieretti, M. V., Conti, G., Staver, A.C., Aquino, S., Cornelissen, J.H.C., 2013. New handbook for standardised measurement of plant functional traits worldwide. Australian Journal of Botany 61, 167–234.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., R Core Team, 2016. nlme: Linear and Nonlinear Mixed Effects Models.

Pinheiro, J.C., Bates, D.M., 2000. Linear Mixed-Effects Models: Basic Concepts and Examples, in: Mixed-Effects Models in S and S-PLUS. Springer-Verlag, New York, pp. 3–56.

Poorter, L., Bongers, F., 2006. Leaf traits are good predictors of plant performance across 53 rain forest species. Ecology 87, 1733–1743.

Pulford, I.D., Watson, C., 2003. Phytoremediation of heavy metal-contaminated land by trees - A review. Environment International 29, 529–540.

R Core Team, 2016. R: A Language and Environment for Statistical Computing.

Revelle, W., 2017. psych: Procedures for Personality and Psychological Research.

Rutten, G., Gómez-Aparicio, L., 2018. Plant-soil feedbacks and root responses of two Mediterranean oaks along a precipitation gradient. Plant and Soil 424, 221–231.

Tabatabai, M. a., Bremner, J.M., 1969. Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biology and Biochemistry 1, 301–307.

Tabatabai, M.A., 1982. Soil Enzymes, in: Page, A.L., Miller, E.M., Keeney, D.R. (Eds.), Methods of Soil Analysis. Part 2. Chemical and Microbiological Properties. Soil Science Society of America, Inc.and American Society of Agronomy, Inc., Madison, pp. 903–947.

Tibbett, M., Sanders, F.E., 2002. Ectomycorrhizal symbiosis can enhance plant nutrition through improved access to discrete organic nutrient patches of high resource quality. Annals of Botany 89, 783–789.

van der Heijden, M.G.A., Martin, F.M., Selosse, M.-A., Sanders, I.R., 2015. Mycorrhizal ecology and evolution: the past, the present, and the future. New Phytologist 205, 1406–1423.

van der Heijden, M.G.A., Scheublin, T.R., 2007. Functional traits in mycorrhizal ecology: their use for predicting the impact of arbuscular mycorrhizal fungal communities on plant growth and ecosystem functioning. New Phytologist 174, 244–250.

van der Putten, W.H., Bardgett, R.D., Bever, J.D., Bezemer, T.M., Casper, B.B., Fukami, T., Kardol, P., Klironomos, J.N., Kulmatiski, A., Schweitzer, J.A., Suding, K.N., Van de Voorde, T.F.J., Wardle, D.A., 2013. Plant-soil feedbacks: the past, the present and future challenges. Journal of Ecology 101, 265–276.

Van Tichelen, K.K., Colpaert, J. V., Vangronsveld, J., 2001. Ectomycorrhizal protection of Pinus sylvestris against copper toxicity. New Phytologist 150, 203–213.

Wardle, D.A., Bardgett, R.D., Klironomos, J.N., Setälä, H., Van Der Putten, W.H., Wall, D.H., 2004. Ecological linkages between aboveground and belowground biota. Science 304, 1629–1633.

Weemstra, M., Mommer, L., Visser, E.J.W., van Ruijven, J., Kuyper, T.W., Mohren, G.M.J., Sterck, F.J., 2016. Towards a multidimensional root trait framework: a tree root review. The New Phytologist 211, 1159–1169.

Wen, Z., Shi, L., Tang, Y., Shen, Z., Xia, Y., Chen, Y., 2017. Effects of Pisolithus tinctorius and Cenococcum geophilum inoculation on pine in copper-contaminated soil to enhance phytoremediation. International Journal of Phytoremediation 19, 387–394.

White, T.J., Bruns, T., Lee, S., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, in: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, Inc., New York.

Wickham, H., 2016. ggplot2: elegant graphics for data analysis. Springer-Verlag, New York.

Wong, M.., 2003. Ecological restoration of mine degraded soils, with emphasis on metal contaminated soils. Chemosphere 50, 775–780.

Wright, I.J., Reich, P.B., Westoby, M., Ackerly, D.D., Baruch, Z., Bongers, F., Cavender-Bares, J., Chapin, T., Cornellssen, J.H.C., Diemer, M., Flexas, J., Garnier, E., Groom, P.K., Gulias, J., Hikosaka, K., Lamont, B.B., Lee, T., Lee, W., Lusk, C., Midgley, J.J., Navas, M.L., Niinemets, Ü., Oleksyn, J., Osada, H., Poorter, H., Pool, P., Prior, L., Pyankov, V.I., Roumet, C., Thomas, S.C., Tjoelker, M.G., Veneklaas, E.J., Villar, R., 2004. The worldwide leaf economics spectrum. Nature 428, 821–827.

Zirbel, C.R., Bassett, T., Grman, E., Brudvig, L.A., 2017. Plant functional traits and environmental conditions shape community assembly and ecosystem functioning during restoration. Journal of Applied Ecology 54, 1070–1079.

Zuur, A.F., 2009. Mixed effects models and extensions in ecology with R. Springer.

Zuur, A.F., Ieno, E.N., Elphick, C.S., 2010. A protocol for data exploration to avoid common statistical problems. Methods in Ecology and Evolution 1, 3–14.

7.7 Supplementary material

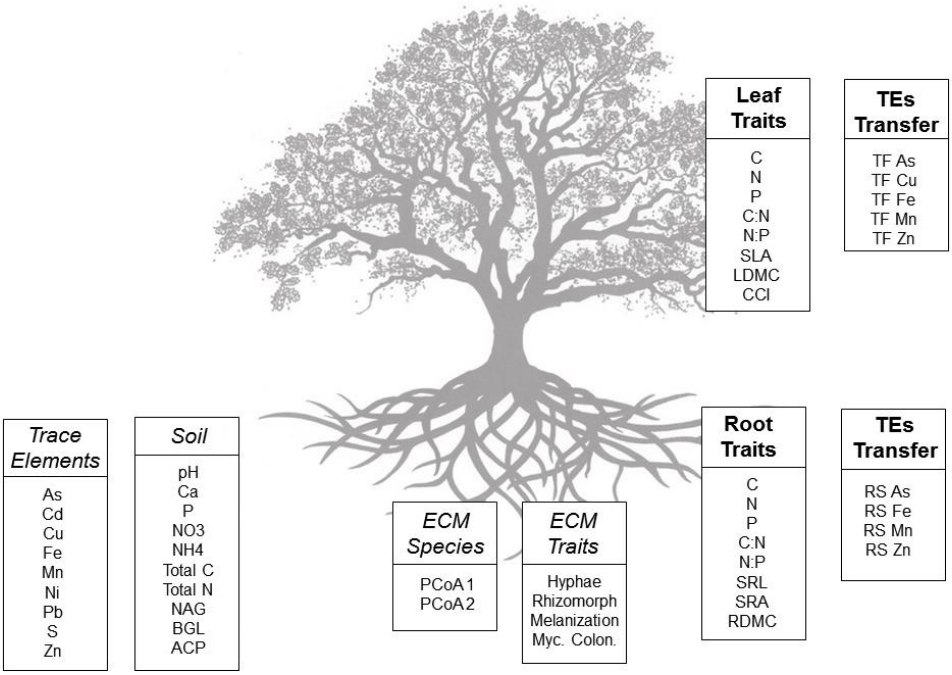


Figure S7.1 Diagram of the predictor (trace elements and soil) and response (trace element transfer, root traits and leaf traits) variables analysed in linear mixed models. NAG: N-acetyl-glucosaminidase; BGL: beta-glucosidase; ACP: acid phosphatase; PCoA: principal coordinate analysis; Myc. Colon.: ectomycorrhizal colonization; SRL: specific root length; SRA: specific root area; RDMC: root dry matter content; RS: soil-to-root; SLA: specific leaf area; LDMC: leaf dry matter content; CCI: chlorophyll content index; TF: translocation factor.

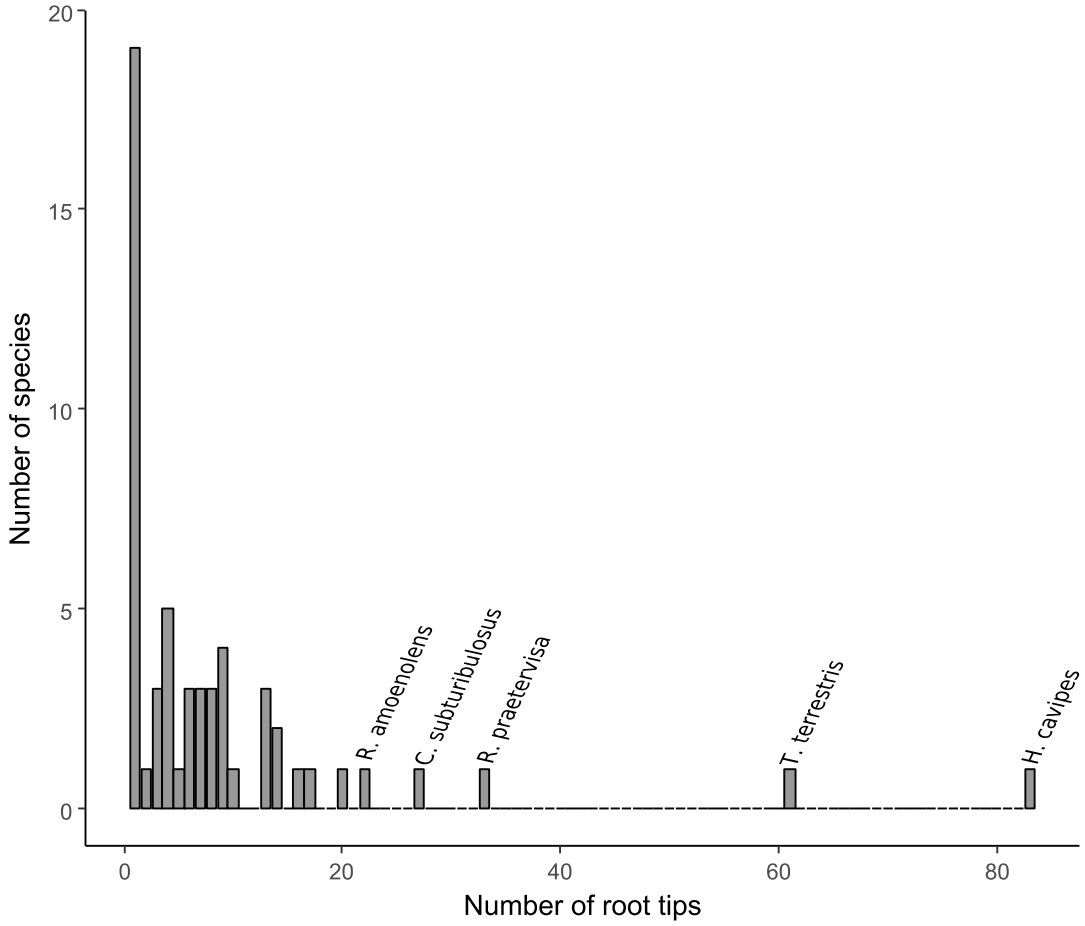


Figure S7.2 Species abundance distribution plot with the most abundant species taxa names.

Table S7.1 Principal Coordinate Analysis (PCoA) scores per species for axis 1 and axis 2 and significance species through permutation test (p<0.05*, p<0.01**, p<0.001***).

Species	**PCoA1**	**PCoA2**
Astraeus_hygrometricus	0.093977	-0.18803
Cenococcum_geophyllum	0.097981	0.066661
*Cortinarius_belleri**	-0.13339	-0.25971
Cortinarius_subbalaustinus	-0.18172	-0.04428
Cortinarius_subturibulosus	-0.24461	-0.10551
Entoloma_inusitatum	-0.06256	0.087172
*Geopora_cervina**	0.288934	0.070499
Geopora_sp._1	0.053234	-0.0107
*Hebeloma_cavipes****	-1.30106	-1.09395
Hebeloma_cistophilum	-0.03011	0.042879
Hymenogaster_griseus	0	0
*Inocybe_curvipes****	-0.16979	0.305908
Inocybe_griseovelata	0.196	0.032404
Inocybe_jacobi	-0.044	0.133281
Inocybe_praetervisa	-0.01739	0.024756
Inocybe_squamata	-0.03771	-0.07163
Laccaria_laccata	-0.09847	0.088341
Lactarius_sp._1	-0.00306	-0.06598
Lactarius_sp._2	-0.00306	-0.06598
Melanogaster_vittadinii	-0.02954	-0.01252
Peziza_michelii	0.149071	0.04692
Pezizaceae_sp.	-0.044	0.133281
Pisolithus_arhizus	0	0
Pisolithus_tinctorius	-0.10681	0.083641
Pustularia_sp.	0.245988	-0.16262
Pyronemataceae_sp._1	0	0

Pyronemataceae_sp._2	0.11051	0.002073
Pyronemataceae_sp._3	0.022166	-0.01164
Russula_amoenolens	0.089316	-0.24717
Russula_ilicis	-0.1255	0.008641
*Russula_insignis**	0.324606	0.067866
*Russula_praetervisa**	0.259963	-0.54192
Russula_sp.	-0.02916	-0.079
Scleroderma_cepa	-0.13574	0.064222
Scleroderma_meridionale	-0.04719	-0.0805
Scleroderma_sp._1	-0.05635	0.041911
Scleroderma_verrucosum	-0.05626	0.095941
*Thelephora_terrestris***	-1.07671	1.225205
Tomentella_castanea	0.212718	0.074104
Tomentella_ferruginea	0.162295	0.056792
Tomentella_lilacinogrisea	-0.06531	-0.12407
Tomentella_sp._1	0.063847	0.019277
Tomentella_sp._10	0.061665	0.020257
Tomentella_sp._2	-0.10581	0.010034
Tomentella_sp._3	-0.01739	0.024756
Tomentella_sp._4	0.123329	0.040513
Tomentella_sp._5	0.174732	0.003278
*Tomentella_sp._6**	0.405988	0.111256
Tomentella_sp._8	0.222298	-0.08937
Tomentella_sp._9	-0.04021	-0.06164
Trichophaeae_sp.	0.268088	0.055364
Tuber_oligospermum	-0.03771	-0.07163
Tuber_sp._1	0	0
Tuber_sp._2	0.14473	0.008194
Tuberaceae_sp._1	0.136643	-0.02444

Table S7.2 Mean and SE of soil (0-20 cm depth) chemistry, enzyme activities and trace elements total and available concentrations. Root and leaf nutrient and trace element concentrations and morphological traits. One-way ANOVA statistic F and p value. Different letters (a, b, c) represent a significant difference between sites. Lower Guideline Values (LGV) for contaminated soils are according to Ministry of the Environment Finland (2007).

			Sites				ANOVA	
Nutrition			**UN**	**US**	**CN**	**CS**	**F**	***p***
pH		Soil	4.84±0.23**c**	6.97±0.15**a**	6.26±0.13**b**	7.33±0.03**a**	51.48	<**0.001**
Ca (g kg^{-1})		Soil	1.89±0.27**b**	4.89±0.09**a**	2.19±0.52**b**	3.24±0.41**b**	13.39	<**0.001**
(g kg^{-1})		Root	10.7±1.9**c**	25.2±2.6**a**	10.9±1.4**bc**	17.9±1.3**b**	13.56	<**0.001**
(g kg^{-1})		Leaf	4.87±0.51	7.84±1.13	6.32±0.73	7.21±0.51	2.83	0.052
P (mg kg^{-1})		Soil	12.72±1.28	8.12±0.88	10.38±1.71	17.17±4.75	0.72	0.547
(g kg^{-1})		Root	0.63±0.07	1.08±0.18	0.70±0.06	0.87±0.10	6.40	0.094
(g kg^{-1})		Leaf	0.96±0.03	0.99±0.05	1.00±0.07	1.12±0.07	1.59	0.210
NH_4 (mg kg^{-1})		Soil	4.77±0.34**a**	2.87±0.17**b**	5.07±0.57**a**	3.49±0.28**b**	10.55	<**0.001**
NO_3 (mg kg^{-1})		Soil	4.78±1.35**a**	2.49±0.45**a**	2.64±0.47**a**	1.21±0.19**b**	4.27	**0.011**
OC (%)		Soil	1.66±0.17**a**	1.07±0.06**bc**	1.42±0.13**ab**	0.85±0.07**c**	9.38	<**0.001**
(%)		Root	43.09±0.92**ab**	39.82±0.97**b**	43.62±1.07**a**	42.42±0.43**ab**	3.65	**0.021**
(%)		Leaf	52.12±1.30**a**	52.58±1.13**a**	47.48±0.20**b**	47.20±0.63**b**	9.88	<**0.001**
N (%)		Soil	0.16±0.02**a**	0.11±0.00**b**	0.15±0.01**a**	0.10±0.01**b**	10.65	<**0.001**
(%)		Root	0.39±0.03	0.37±0.06	0.35±0.04	0.24±0.02	2.32	0.091
(%)		Leaf	1.34±0.03	1.29±0.04	1.33±0.05	1.33±0.07	0.23	0.877
C:N		Soil	10.48±0.24**a**	9.93±0.20**a**	9.27±0.41**ab**	8.18±0.43**b**	8.63	<**0.001**
		Root	118.7±11.6**b**	131.5±16.0**ab**	141.6±14.8**ab**	196.0±22.0**a**	3.38	**0.028**
		Leaf	38.97±1.33	41.31±1.86	36.07±1.31	36.36±2.32	1.96	0.137
N:P		Root	6.37±0.55**a**	3.67±0.43**bc**	5.08±0.63**ab**	3.06±0.41**c**	8.34	<**0.001**
		Leaf	14.09±0.51	13.14±0.51	13.84±1.00	12.18±0.72	1.44	0.247
Enzyme Activities (µmol PNF g^{-1} h^{-1})								
NAG		Soil	0.332 ±0.045**b**	0.193±0.014**c**	0.623±0.055**a**	0.455±0.047**ab**	18.52	<**0.001**
BGL		Soil	0.94±0.15**b**	1.13±0.11**b**	1.85±0.18**a**	0.94±0.09**b**	9.64	<**0.001**
ACP		Soil	1.47±0.15**b**	0.64±0.03**c**	2.26±0.24**a**	0.97±0.21**bc**	16.29	<**0.001**
Total Trace Elements (mg kg^{-1})								
	LGV							
As	50	Soil	161.83±21.71**a**	40.39±4.98**b**	18.03±1.27**c**	13.52±1.09**c**	97.26	<**0.001**
		Root	18.62±5.06**a**	5.23±0.74**ab**	3.48±0.70**b**	2.68±0.10**b**	27.65*	<**0.001**
		Leaf	0.41±0.10**b**	0.78±0.10**ab**	1.20±0.14**a**	1.13±0.21**a**	6.41	**0.001**
Cd	10	Soil	0.68±0.11**a**	0.67±0.07**a**	0.21±0.03**b**	0.02±0.01**c**	43.62	<**0.001**
		Root	1.78±0.15**a**	1.20±0.18**b**	0.82±0.11**b**	0.40±0.03**c**	26.57	<**0.001**
		Leaf	0.18±0.05	0.08±0.01	0.08±0.02	0.09±0.01	2.36	0.088

Cu	150	Soil	192.55±7.82**a**	58.15±5.70**b**	40.54±4.46**b**	18.69±1.72**c**	211	**<0.001**
		Root	86.08±8.13**a**	35.09±6.51**b**	29.86±8.75**b**	12.25±1.47**c**	28.78	**<0.001**
		Leaf	5.65±0.35**bc**	4.75±0.15**c**	6.98±0.30**a**	6.09±0.33**ab**	10.16	**<0.001**
Fe		Soil	40475±2136**a**	21968±565**c**	27522±935**ab**	22796±1498**bc**	27.57*	**<0.001**
		Root	4769±707**a**	3238±399**b**	3035±159**b**	1993±133**c**	12.93	**<0.001**
		Leaf	64.38±6.63	54.05±6.65	80.25±14.73	63.64±11.47	4.36*	0.225
Mn		Soil	391.5±39.5**b**	414.8±15.4**b**	851.9±29.6**a**	486.4±47.8**b**	37.09	**<0.001**
		Root	135.2±15.9**ab**	92.4±17.7**b**	198.2±27.9**a**	116.4±15.9**ab**	5.26	**0.004**
		Leaf	458±111**b**	36.84±6.31**c**	1298±219**a**	451±85**b**	16.55	**<0.001**
Ni	100	Soil	13.01±0.70**b**	14.60±0.44**b**	21.69±1.04**a**	15.73±1.23**b**	17.44	**<0.001**
		Root	5.51±0.43**ab**	4.48±0.51**b**	7.23±0.75**a**	4.48±0.43**b**	5.62	**0.003**
		Leaf	1.12±0.17**a**	0.14±0.04**b**	1.87±0.36**a**	0.88±0.20**a**	10.12	**<0.001**
Pb	200	Soil	274.40±37.54**a**	76.66±8.26**b**	57.57±6.87**b**	19.82±1.14**c**	89.89	**<0.001**
		Root	36.63±8.96**a**	12.09±3.06**b**	18.01±4.19**b**	3.32±0.68**c**	27.48	**<0.001**
		Leaf	0.36±0.04**b**	0.30±0.07**b**	0.82±0.17**a**	0.36±0.04**b**	6.40	**0.001**
S		Soil	3117±409**a**	706±94**b**	166±18**c**	98.5±7.6**c**	123.3	**<0.001**
		Root	0.085±0.010**a**	0.079±0.010**a**	0.061±0.012**ab**	0.044±0.002**b**	21.00	**<0.001**
		Leaf	0.096±0.003	0.090±0.003	0.087±0.003	0.090±0.003	1.67	0.191
Zn	250	Soil	228.99±29.61**a**	229.65±21.54**a**	96.93±9.25**b**	44.43±3.71**c**	66.39	**<0.001**
		Root	129.6±13.7**a**	86.41±17.94**ab**	56.47±12.34**b**	15.26±1.06**c**	14.07	**<0.001**
		Leaf	48.63±6.33**a**	20.61±1.63**b**	23.19±1.60**ab**	20.11±1.10**b**	16.19*	**0.001**
Available Trace Elements (mg kg^{-1})								
Cd		Soil	0.113±0.029**a**	0.003±0.001**b**	0.025±0.007**ab**	0.003±0.001**b**	26.01*	**<0.001**
Cu		Soil	1.70±0.60**a**	0.082±0.004**b**	0.072±0.011**b**	0.044±0.005**b**	29.04*	**<0.001**
Fe		Soil	2.56±0.30**a**	1.09±0.06**b**	1.37±0.25**b**	1.12±0.14**b**	10.84	**<0.001**
Mn		Soil	19.33±5.30**a**	0.08±0.01**c**	22.32±6.00**a**	1.44±0.79**b**	29.41	**<0.001**
Ni		Soil	0.372±0.082**a**	0.049±0.007**b**	0.182±0.042**a**	0.062±0.005**b**	17.66	**<0.001**
S		Soil	329.7±141.1**a**	11.20±1.74**b**	9.25±2.48**bc**	4.73±0.66**c**	19.02	**<0.001**
Zn		Soil	14.35±3.58**a**	0.28±0.16**c**	2.52±0.95**b**	0.12±0.02**c**	35.21	**<0.001**
Morphological Traits								
SRA (m^2 kg^{-1})		Root	10.09±0.65	9.77±0.72	9.02±0.48	9.13±0.34	0.83	0.489
SRL (m g^{-1})		Root	5.14±0.36	5.31±0.43	4.62±0.35	5.42±0.30	0.95	0.426
SLA (m^2 kg^{-1})		Leaf	4.83±0.16**ab**	4.48±0.15**b**	4.91±0.10**ab**	5.03±0.13**a**	2.98	**0.044**
RDMC(mg g^{-1})		Root	430.5±20.6	410.3±17.4	440.0±11.8	445.9±7.9	1.05	0.382
LDMC(mg g^{-1})		Leaf	53.03±0.48	53.94±0.54	53.82±0.74	52.29±0.43	1.89	0.149
CCI (SPAD unit)		Leaf	47.59±1.07	50.46±0.87	47.31±1.25	46.63±2.03	1.51	0.229

8. ELEMENTOS TRAZA E ISÓTOPOS EN SETAS SILVESTRES

Resumen

Los hongos del suelo juegan un importante papel en el funcionamiento de los ecosistemas terrestres y, en particular, en la remediación de suelos degradados. La contribución de los hongos en los ciclos del carbono y nutrientes, junto con su capacidad para movilizar los elementos traza del suelo, ha sido reconocida y estudiada. Sin embargo, la importancia del estilo de vida de los hongos en estas funciones aún no se conoce en profundidad. Este estudio explora las relaciones suelo-hongo a través de dos setas silvestres, la especie ectomicorrícica *Laccaria laccata* y la especie saprótrofa *Volvopluteus gloiocephalus*. Tanto las setas como los suelos, en una zona contaminada tras un vertido minero, sobre los que se desarrollan, fueron evaluados. El análisis isotópico mostró que las setas de *Laccaria laccata* estaban enriquecidas en ^{15}N en comparación con las setas de *Volvopluteus gloiocephalus*, posiblemente por la transferencia de nitrógeno poco enriquecido en ^{15}N a la planta hospedadora. Además, las setas de *Laccaria laccata* mostraron unos valores $\delta^{13}C$ que indicaban que el carbono podría provenir del hospedador mientras que los valores $\delta^{13}C$ de las setas de *Volvopluteus gloiocephalus* fueron similares a los del suelo. Ambas especies mostraron una alta bioacumulación de Cd y Cu en sus cuerpos fructíferos. El consumo humano de estas setas podría representar un riesgo de toxicidad por la elevada concentración de Cd en ambas setas. Esta alta capacidad para bioacumular elementos traza de los hongos indica que existe un potencial de investigación del papel de "micorremediación" en zonas contaminadas.

Abstract

Fungi play a key role in the functioning of soil in terrestrial ecosystems, and in particular in the remediation of degraded soils. The contribution of fungi to carbon and nutrient cycles, along with their capability to mobilise soil trace elements, is well-known. However, the importance life history strategy for these functions has not yet been thoroughly studied. This study explored the soil-fungi relationship of two wild edible fungi, the ectomycorrhizal *Laccaria laccata* and the saprotroph *Volvopluteus gloiocephalus*. Fruiting bodies and surrounding soils in a mine-spill contaminated area were evaluated. Isotope analyses revealed *Laccaria laccata* fruiting bodies were ^{15}N-enriched when compared to *Volvopluteus gloiocephalus*, likely due to the transfer of ^{15}N-depleted nitrogen to their host plant. Moreover, *Laccaria laccata* fruiting bodies $\delta^{13}C$ values were closer to host plant values than surrounding soil, while *Volvopluteus gloiocephalus* matched $\delta^{13}C$ composition to that of the soil. Fungal species presented a high bioaccumulation and concentrations of Cd and Cu in their fruiting bodies. Human consumption of these fruiting bodies may represent a toxicological risk due to their elevated Cd concentrations. The high bioaccumulation capacity found in the studied fungal species indicates that further investigation of the "mycoremediation" potential of fungi is of interest.

8.1. Introduction

Fungi play a key role in the functioning of terrestrial ecosystems due to their diverse capabilities and relevance in nutrient cycling. Fungal functionality is directly related to their wide life history strategies, being classified as saprobes, parasites, pathogens and/ or symbionts. Saprobes (or saprotrophs) are free-living fungi that obtain nutrients mostly from decomposing soil organic matter and, due to this ability, are considered the most important decomposers in terrestrial ecosystems. Symbiotic mycorrhizal fungi also play a key role in nutrient cycling (Hobbie et al., 1999a; Phillips et al., 2013). Mycorrhizal fungi are able to take up nutrients and water, and transfer them to the host plant, in exchange for photosynthates (Lindahl and Tunlid, 2015; Smith and Read, 2008). Ectomycorrhizal fungi may also mobilise soil trace elements enhancing their transfer from soil to plant and/or fungal tissues. Saprotrophic fungi dominate the litter layer in soils and acquire C and nutrients in the form of organic substances by enzymatic decomposition (Sinsabaugh et al., 2008). Symbiotic ectomycorrhizal fungi are normally found in deep soil layers (well-degraded litter and humus) with a high nutrient content but low C content, as they are not C-limited (Lindahl et al., 2007; McGuire et al., 2013). Ectomycorrhizal fungi are highly relevant in forests, including Mediterranean ones, as they associate with numerous tree and shrubs species (Courty et al., 2010). Previous studies found independent roles between ectomycorrhizal and saprotrophic fungi, with ectomycorrhizal fungi being able to degrade recalcitrant N-rich compounds, while saprotrophic fungi being able to degrade labile C-rich biopolymers (Talbot et al., 2013). Although ectomycorrhizal fungi have a lower ability to decompose organic matter than saprobes, they can obtain resources along the biotrophy–saprotrophy continuum (Koide et al., 2008; Read et al., 2004). For example, limited supply of plant photosynthate may increase enzyme production for obtaining labile carbohydrates from soil organic matter by ectomycorrizal fungi (Courty et al., 2010, 2007). Ecological consequences may arise depending on the situation of the fungus within the continuum as, for example, ectomycorrhizal fungi fructification may fail if separated from plant roots (Ishida et al., 2008; Koide et al., 2008). Therefore, the spectrum of life history strategies implies that fungi differ in their roles and in their effects on ecosystem functioning.

Different mechanisms of resource acquisition, loss and cycling in fungi can be inferred by studying stable isotopes in fungal fruiting bodies (mushrooms) (Agerer et al., 2012;

Hobbie et al., 2004; Hobbie and Horton, 2007). Previous studies found that ectomycorrhizal fungal sporocarps present higher $\delta^{15}N$ values and lower $\delta^{13}C$ values than saprotrophic ones (Hobbie et al., 2001, 1999a; Hobbie and Horton, 2007; Mayor et al., 2009). Ectomycorrhizal fungi compete for N against saprotrophic fungi and bacteria, and they discriminate strongly against ^{15}N (Hobbie and Högberg, 2012). Ectomycorrhizal fungi are known for their N isotope fractionation in the plant-soil system, as fungi retain ^{15}N-enriched N while ^{15}N-depleted N is transferred to the host plant. Therefore, analysing and comparing $\delta^{13}C$ and $\delta^{15}N$ values in fruiting bodies and underlying soils could help us to understand differences between saprotrophic and mycorrhizal strategies for resource acquisition (Hobbie et al., 1999a).

Fungi have been extensively studied in trace element contaminated areas as they are able to develop mechanisms to tolerate high trace element concentrations (see Bellion et al. 2006 for ectomycorrhizal fungi (Bellion et al., 2006b)). Fungi may reduce the entry of trace element concentrations into cells by a range of extracellular mechanisms, such as chelation by excreted ligands, cell-wall binding and enhanced efflux. Despite this, some amount of trace elements may still enter cells, but their toxicity can be reduced by other intracellular mechanisms, such as chelation by peptides and subcellular compartmentation (Baldrian, 2010). In basidiomycetes, the mechanisms of response to trace element contamination are diverse and species dependent (Baldrian, 2010; Godbold et al., 1998). For example, the response of *Agaricus* to copper (Cu) is to produce metallothionein, while the response to cadmium (Cd) is to induce the production of mycophosphatin (Baldrian, 2010).

Due to these protective mechanisms of fungi, many studies have focused on analysing the accumulation of trace elements into aboveground fungal biomass, as human consumption of edible mushrooms causes concern. In some trace element contaminated soils, a high accumulation of trace elements has been found in edible mushrooms growing there. In a silver-mining area of Czech Republic, high concentrations of trace elements were found in edible mushrooms: 149 mg kg^{-1} dry matter of Cd in *Agaricus silvaticus*, 12.9 mg kg^{-1} dry matter of mercury (Hg) in *Lepista nuda* and 16.2 mg kg^{-1} dry matter of lead (Pb) in *Lycoperdon perlatum* (Svoboda et al., 2006). In another study, in a smelter area of Austria, the collected mushrooms had , elevated concentrations of zinc (Zn) (up to 777 mg kg^{-1}) and Cd (up to 127 mg kg^{-1}) (Krpata et al., 2009).

Besides the numerous studies published, the relationships between trace element concentrations in soils and in fungal fruiting bodies are not always consistent and remain unclear. Some studies in the literature have found that fungal species may behave as bioindicators of trace elements, as concentrations in fungal fruiting bodies and their substrates correlated (Alonso et al., 2003; Malinowska et al., 2004; Proskura et al., 2017). On the other hand, separate studies reported that elemental concentrations in fungi and soil did not correlate; they could have the ability to exclude trace elements or, at the contrary, to accumulate them into their biomass to levels well above (but not necessarily correlated to) the corresponding concentrations in soil (Colpaert et al., 2011; Krpata et al., 2009). This controversy is also affected by the soil extraction method selected to determine trace element concentration (Buscaroli, 2017). Most of the fungal studies used soil total fractions (including residual unavailable soil fractions) instead of readily or potentially available fractions(Falandysz et al., 2016; Melgar et al., 2016; Mleczek et al., 2013). However, the use of soil "available" fractions to compare with sporocarp concentrations is recommended (Komárek et al., 2007; Krpata et al., 2009).

In order to improve our understanding of the soil-fungi relationship, the selection and analysis of the multiple factors that may explain the mushroom trace element accumulation patterns is required. First, in multi-element contaminated substrates, interactions among trace elements in soil and fungal biomass may further complicate our ability to foresee bioaccumulation patterns. In conditions of high concentrations of soil trace elements there may be a competitive interaction between some elements, for example between Cd and Zn. Some ectomycorrhizal species have a preferential uptake of Zn (essential element), through a competitive inhibition of Cd uptake (toxic element) (Colpaert and Van Assche, 1992; Hartley et al., 1997b). Second, there are many environmental factors related to fungal accumulation of trace elements (Ali et al., 2017). Soil variables, such as soil organic matter, pH and iron (Fe), manganese (Mn) and aluminium (Al) (oxyhydr)oxides (among others) may explain fungal trace element accumulation, as they have a profound influence on trace element availability in the soil solution. Third, trophic differences among saprotrophs and ectomycorrhizal fungi can be related with variations in trace elements concentrations in both fruiting bodies and underlying soils, due to their different roles in soil organic matter decomposition and C and nutrient cycles(Talbot et al., 2013). Therefore, the concentration of trace elements in the soil is not the only determinant on their accumulation pattern in fungal fruiting bodies, as the soil-fungi relationship is very complex. The Bioconcentration Factor (BCF)

(concentration in sporocarp divided by concentration in soil) is commonly calculated to elucidate this intricate translocation of trace elements from surrounding soil to fruiting bodies (Ali et al., 2017; Tyler, 1982). This BCF is therefore a very useful ratio which allows us to understand how different fungal species, fructifying in similar environmental conditions, may differ in trace element uptake and accumulation.

This study is of interest within the overall context of soil remediation processes and arises from the necessity of monitoring trace element concentrations in edible mushrooms growing in a contaminated site, as well as to elucidate the differential response of saprotrophic and ectomycorrhizal fungi to soil contamination by trace elements. We analysed the dynamics of nutrients and trace elements in two wild edible fungi species: *Laccaria laccata* (Scop.) Cooke, which was associated with stone pine and grey-leaved cistus by ectomycorrhizal symbiosis in the study area, and *Volvopluteus gloiocephalus* (DC.) Vizzini, Contu & Justo, saprotrophic fungi, fructifying in grassland soils. In a trace element contaminated and remediated ecosystem, we selected these fungal species as both were edible and fructified in the study area in the same period; in addition, they have contrasting life history strategy.

The specific objectives of this study were: 1) Determining fungal nutritional sources using C and N isotope composition; 2) Determining the accumulation capacity of trace elements in fungal fruiting bodies and the relations with soil properties; 3) Evaluating the potential toxicological risk of fruiting bodies (mushrooms) for human consumption.

8.2. Materials and methods

8.2.1. Study area

The study was conducted in the Guadiamar Green Corridor (Seville, SW Spain; 37º 23.165´ N, 6º 13.668´ W) which is a public area managed by the Spanish Regional Government. The study area was located in the contaminated North site. See the general description of the study site in chapter 3.The study area is one of these degraded areas with high toxicity risks due to the naturally sandy and acidic soil, with a limited capacity of soils to buffer against acid drainage caused by the remnants of mining residues.

8.2.2. Study fungal species

The two selected species in this study were *Laccaria laccata*, which form ectomycorrhizae, and *Volvopluteus gloiocephalus*, which is a saprobe (Fig. 5). Both species belong to the Basidiomycota division and Agaricales order, but are classified in different families. *Laccaria laccata* belongs to the Hydnangiaceae family and *Volvopluteus gloiocephalus* to the Pluteaceae family. *Laccaria laccata* is distributed broadly in the Northern Hemisphere and has small, red-orange fruiting bodies. This fungal species has a wide ectomycorrhizal host potential, a low specificity and it is characteristic of young stands of trees (Dix and Webster, 1995). *Volvopluteus gloiocephalus* is distributed in Europe and North America and has bigger fruiting bodies than *Laccaria laccata* with white or grey-brown colours (Justo et al., 2011).

The specimens of fruiting bodies collected for this work were registered in the University of Seville Herbarium to the codes SEV-C 51 for *Laccaria laccata* and SEV-C 50 for *Volvopluteus gloiocephalus.* Molecular analyses were carried out to confirm the species morphological determination and to identify potential ecotypes. Sequences are available in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) under accession numbers MK616245 - MK616246 for *Laccaria laccata* and MK616344 - MK616347 for *Volvopluteus gloiocephalus*.

8.2.3. Sampling design

In December 2016, a field sampling was conducted in order to evaluate the wild growing edible fungal fruiting bodies that fructified in the remediated soils of this area. Fruiting bodies were sampled in the locations where they fructified, naturally occurring in separated habitats. *Laccaria laccata* sporocarps were found close to the ectomycorrhizal tree, *Pinus pinea*, and the shrub, *Cistus albidus*. In contrast, *Volvopluteus gloiocephalus* sporocarps were found in adjacent treeless areas covered by different herbaceous species. Therefore, sampling locations were dependent on the distribution of these habitats in the study area (Fig. S2). For each species, ten points and at least three sporocarps per point were sampled. Soil surrounding each sporocarp was taken (top 0-5 cm depth) with an

auger from at least five locations within each point to make a composite sample per point. Sporocarps and soils were kept refrigerated until being processed the day after sampling.

8.2.4. Soil analyses

Soil samples (top 0-5 cm depth; 100 g fresh weight) were air-dried and sieved to < 2 mm for chemical analysis, where soil pH was measured in a 1:2.5 soil-water suspension after 30 min of shaking. Bray 1 method was selected to determine soil P in these acidic soils (Kuo, 1996). Available K was extracted with 1 M ammonium acetate and determined by multiparametric Bran-Luebbe autoanalyser. Pseudo-total concentrations of trace elements (As, Cd, Co, Cu, Fe, Mn, Ni, Pb, S and Zn), Ca and Mg were extracted by digestion of soils, ground to < 60 µm, with *aqua regia* (1:3 v/v conc. HNO_3/ HCl) in a Digiprep MS block digester (SPS Science) for 2 h at 110 ºC. Trace elements (As, Cd, Co, Cu, Fe, Mn, Ni, Pb, S and Zn) were extracted from < 2 mm sieved soils with a 0.01 M $CaCl_2$ solution to estimate their "bioavailability" (Houba et al., 2000). Extracts after digestion with *aqua regia* or $CaCl_2$, were determined by inductively coupled plasma optical emission spectrophotometry (ICP-OES) with Varian ICP 720-ES. The quality of the analyses was assessed using the reference sample ERM-CC141 (loam soil) and recoveries from 93.8% to 100.4% were obtained. Arsenic extracted by $CaCl_2$ presented concentrations below detections limits (0.005 mg kg-1), and its "availability" is underestimated by this method.

8.2.5. Chemical composition of fungal fruiting bodies

Fruiting bodies were dry cleaned with a brush and fresh weighed, with some biomass kept at -80 ºC for further species identification by molecular analysis. Sporocarps were dried at 70 ºC for 48 h and stored until analysis. The whole fruiting body (cap, hymenophore and stalk) was analysed as one single sample, as all the parts are usually ingested when consumed by humans. Fruiting bodies macronutrients (Ca, K, Mg and P) and trace elements were extracted by digestion with HNO_3 in a Digiprep MS block digester (SPS Science) for 2 h at 110 ºC and determined by ICP-OES with Varian ICP 720-ES. Arsenic

was determined by ICP-OES with Ion Exchange Hydride Generation (IE-HG-ICP-OES) methodology (European Committee for Standardization, 2008). The quality of the analysis was assessed using the reference sample ID 124 Type Lucerne (*Medicago sativum*) (WEPAL; IPE) for As, with recoveries of 95.9%, and the reference sample INCT-OBTL-5 (tobacco leaves) for the rest of the trace elements, with recoveries from 79.9% to 112.2%.

8.2.6. Isotopic analysis

Total C and N and their isotope composition ($\delta^{13}C$ and $\delta^{15}N$, respectively) in soil and sporocarps were determined by elemental analysis and isotope ratio mass spectrometry (EA/IRMS) system by means of Flash HT Plus elemental analyser coupled to a Delta-V Advantage isotope ratio mass spectrometer via a CONFLO IV interface (Thermo Fisher Scientific, Bremen, DE). The analytical measurement errors were ±0.1‰ for $\delta^{13}C$ and ±0.2‰ for $\delta^{15}N$. Total C was organic C due to the absence of carbonates in the study area (Domínguez et al., 2016).

8.2.7. Data analysis

Mean and standard error (SE) of datasets from *Laccaria laccata* and *Volvopluteus gloiocephalus* sporocarps and respective soils were calculated. The chemical composition of soils and sporocarps of the studied species were compared through a parametric Student's t-tests. Normality assumptions were verified; Lilliefors-corrected Kolmogorov-Smirnov test for normality and Levene test for homogeneity of variance. If the assumptions were not met, the data was Box-Cox transformed. If this transformation did not meet the assumptions, a non-parametric Mann-Whitney test was subsequently performed.

To evaluate the level of contamination, the values of soil pseudo-total concentration of trace elements were compared with background values (BV) for the South Portuguese Zone (Galán and Romero, 2008). The reference level (percentile 90), which is the maximum value accepted for non-contaminated soils, was used.

Bioconcentration factors of trace elements were calculated as concentration in fruiting bodies divided by pseudo-total concentration in soil underneath (both in mg kg^{-1}) (Ali et al., 2017; Tyler, 1982). To interpret the results, a BCF > 1 suggests that fungi have some transporters (active or passive) that can carry trace elements into fungal biomass resulting in accumulation, while a BCF < 1 may indicate the existence of fungal mechanisms against take up of trace elements (Buscaroli, 2017). In addition, the ratio between trace elements accumulated in the fungal fruiting bodies and those "bioavailable" in the soil (estimated by $CaCl_2$ extraction)was calculated as a functional transfer ratio (Komárek et al., 2007).

To explore relationships among sporocarps BCF and soil variables, a nonmetric multidimensional scaling (NMDS) was performed (Kruskal, 1964). The BCF data was standardised with a Hellinger transformation and selected Euclidean distance metrics, where the number of dimensions was k = 2 with an adequate level of stress of 0.010. Soil variables effects were calculated using a permutation model (with 999 permutations) and fitted using the *envfit* function(Oksanen et al., 2019). In this plot, the length of arrows represents the strength of the correlation and its direction indicates the trend of change in the variable.

To evaluate relationships of trace elements in the soil ($CaCl_2$-extracted fraction) with sporocarps we performed a Pearson´s correlation test, adjusted with Benjamini-Hochberg correction (Benjamini and Hochberg, 1995).

All data analyses were performed utilising the R software package v3.5.1(R Core Team, 2018) using *ggplot2*(Wickham, 2016), *lawstat*(Gastwirth et al., 2019), *MASS*(Venables and Ripley, 2002), *nortest*(Gross and Ligges, 2015), *psych*(Revelle, 2019) and *vegan*(Oksanen et al., 2019) packages.

8.2.8. Evaluation of toxicity risk

Fruiting bodies concentrations of potentially toxic elements (Cd and Pb) in the two fungal species were compared with the regulation limits (in fresh weight) for edible mushrooms of the European Union (European Commission, 2015, 2014). The fruiting bodies concentrations of Cd and Pb were transformed from dry weight to fresh weight by dividing the limits (Cd limited to 1 mg kg^{-1} fresh weight; Pb limited to 0.3 mg kg^{-1} fresh

weight) by the mean fresh weight (*Laccaria laccata* 7.13 g fresh weight; *Volvopluteus gloiocephalus* 5.77 g fresh weight) (Table S8.3).

Fungi minimum daily intake to reach the upper level of the tolerable intake threshold, established by the Joint FAO/WHO Expert Committee on Food Additives (WHO, 2017), were calculated to those trace elements reported in that guideline: As, Cd, Cu, iron (Fe), Pb and Zn. The guideline limits were transformed into provisional maximum tolerable daily intake (PMTDI) and the weights were expressed as kg of fresh weight for a person with 70 kg bodyweight (as proposed by the European Food Safety Authority) (Table S8.2).

8.3. Results

8.3.1. Soil properties

Soil properties in the study area were relatively homogenous for total C and N, available P, total Mg and some trace elements (Tables 8.1 and 8.2). However, there were significant differences between soils underneath fruiting bodies of ectomycorrhizal *Laccaria laccata* and saprotrophic *Volvopluteus gloiocephalus* fungi for other soil variables. Despite the fact that soil pH was acidic in the study area, *Laccaria laccata* underlying soils had lower pH (mean difference of 1.47 units) than *Volvopluteus gloiocephalus* soils (Table 8.1). Available K and total Ca were significantly different in the soils underneath both species, with higher concentrations in *Volvopluteus gloiocephalus* underlying soils (Table 8.1).

Soil pseudo-total arsenic (As), Cu, Fe, nickel (Ni), Pb and sulphur (S) were relatively homogeneous in the study area. However, pseudo-total Cd, cobalt (Co), Mn and Zn differed between fungal species habitats, being significantly higher concentrations in *Volvopluteus gloiocephalus* underlying soils (Table 8.2). Soil "available" ($CaCl_2$-extracted) concentrations of trace elements presented a different trend than pseudo-total values. Soil underneath *Laccaria laccata* sporocarps had significantly higher $CaCl_2$-extracted concentrations for almost all trace elements (Cd, Co, Cu, Mn, Ni and Zn), except for Fe, Pb and S concentrations that were similar in both soils (Table 8.2).

Table 8.1 Main soil chemical properties associated to *Laccaria laccata* and *Volvopluteus gloiocephalus* fungal species. Mean values (± SE) for N = 10. Student's *t*-test statistic and *p* value (in bold $p < 0.05$) are shown. * Data transformation for normality of the residuals.

Soil properties	***Laccaria laccata***	***Volvopluteus gloiocephalus***	***t***	***p***
pH	4.6 ± 0.2	6.05 ± 0.15	-7.00	< **0.001**
C_{total} (%)	0.901 ± 0.152	1.09 ± 0.12	-1.00	0.330
N_{total} (%)	0.169 ± 0.015	0.195 ± 0.010	-1.49	0.157
$P_{available}$ (mg kg^{-1})	11.5 ± 2.7	10.8 ± 0.8	1.22*	0.249
$K_{available}$ (mg kg^{-1})	166 ± 16	272 ± 14	-5.07	< **0.001**
Ca_{total} (mg kg^{-1})	2127 ± 149	3469 ± 130	-6.79	< **0.001**
Mg_{total} (mg kg^{-1})	3014 ± 98	3122 ± 92	-0.81	0.428

Table 8.2 Soil trace elements ($CaCl_2$-extractable and pseudo-total) associated with *Laccaria laccata* and *Volvopluteus gloiocephalus* fungal species. Mean values (± SE) in mg kg^{-1} for N = 10. Student's *t*-test statistic and *p* value (in bold $p < 0.05$). Background values (BV) at percentile 90 (P 90) in South Portuguese Zone soils at depth 0-20 cm in mg kg^{-1} [83]. § Data transformation for normality of the residuals. ◊ Non-parametric Mann-Whitney test (U statistic).

Soil trace elements	***Laccaria laccata***	***Volvopluteus gloiocephalus***	**BV (P 90)**	***t***	***p***
Pseudo-total					
As	142 ± 7	123 ± 15	157	1.12	0.282
Cd	0.630 ± 0.062	1.08 ± 0.05		-5.59	< **0.001**
Co	8.38 ± 0.47	11.2 ± 0.4	34	-4.54	< **0.001**
Cu	174 ± 13	185 ± 8	108	-0.73	0.475
Fe	37222 ± 1187	35973 ± 1902		0.56	0.586
Mn	301 ± 18	418 ± 17		-4.66	< **0.001**
Ni	14.7 ± 0.4	14.9 ± 0.4	62	-0.34	0.737

Pb	227 ± 14	204 ± 26	117	0.77	0.456
S	2421 ± 319	1769 ± 438		-2.07§	0.058
Zn	193 ± 10	263 ± 11	134	-4.67	< **0.001**
$CaCl_2$-extractable					
Cd	0.142 ± 0.019	0.042 ± 0.008		4.91	< **0.001**
Co	0.145 ± 0.038	0.019 ± 0.005		4.51§	< **0.001**
Cu	1.72 ± 0.70	0.253 ± 0.031		-4.68§	< **0.001**
Fe	4.90 ± 0.42	4.30 ± 0.87		0.62	0.544
Mn	15.2 ± 1.9	4.52 ± 0.88		5.19	< **0.001**
Ni	0.354 ± 0.036	0.103 ± 0.015		6.45	< **0.001**
Pb	0.082 ± 0.015	0.083 ± 0.021		-0.06	0.957
S	135 ± 75	74.5 ± 53.2		62◊	0.182
Zn	14.8 ± 1.9	3.12 ± 0.91		5.66	< **0.001**

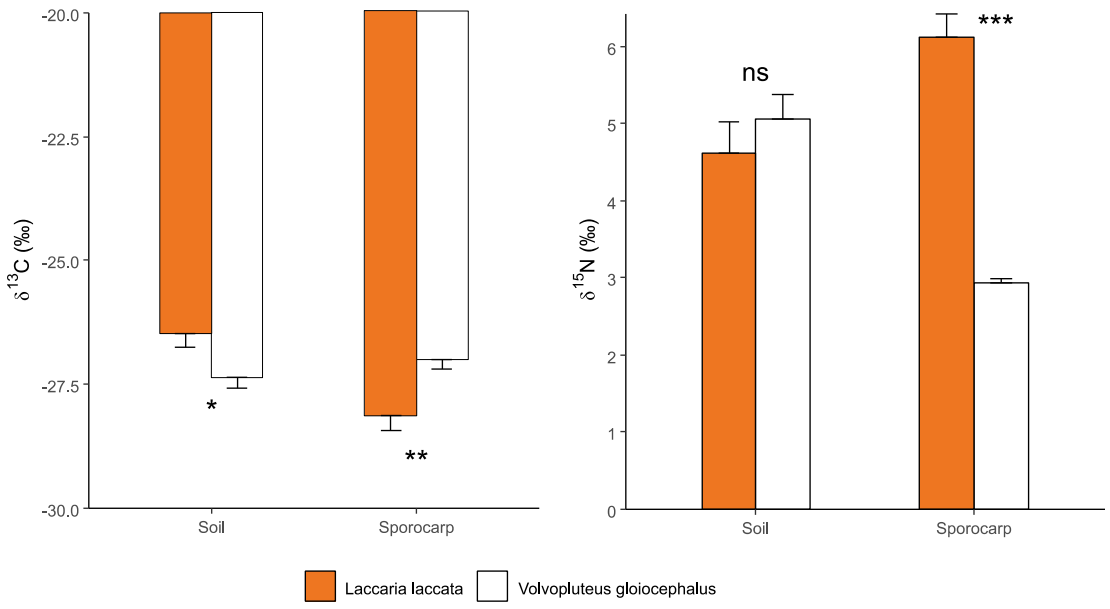
8.3.2. Carbon and nitrogen isotopes in soil and fungal sporocarps

Soil C content was similar for both fungal species, however C isotope composition of these soils was different; *Laccaria laccata* surrounding soils were ^{13}C-enriched compared to *Volvopluteus gloiocephalus* ^{13}C-depleted surrounding soils (Table 8.1 and Fig. 8.1A). Ectomycorrhizal *Laccaria laccata* fungal sporocarps were ^{13}C-depleted relative to saprotrophic *Volvopluteus gloiocephalus* fungi (Fig. 8.1A). *Laccaria laccata* fruiting bodies were ^{13}C-depleted compared to their soil (t = 4.24; p <0.001), whereas *Volvopluteus gloiocephalus* fruiting bodies were not significantly different to their soil (t = -1.31; p = 0.208) (Fig. 8.1A).

Soil N content was similar for both fungal species as well as their N isotope composition (Fig. 8.1B). In contrast to δ^{13}C values, ectomycorrhizal *Laccaria laccata* sporocarps were ^{15}N-enriched in comparison to saprotrophic *Volvopluteus gloiocephalus* sporocarps. Ectomycorrhizal *Laccaria laccata* sporocarps were ^{15}N-enriched compared to the

surrounding soil (t = -2.99; p = 0.009), whereas saprotrophic *Volvopluteus gloiocephalus* fungi were ^{15}N-depleted (t = 6.51; p < 0.001) (Fig. 8.1B).

Figure 8.1 A) Carbon and B) Nitrogen isotope composition in soil and sporocarps of *Laccaria laccata* and *Volvopluteus gloiocephalus* fungal species. Bars represent mean values and error bars represent standard error for N = 10. *P* values resulting from Student's *t*-test are indicated (ns: non-significant; p < 0.05*; p < 0.01**; p < 0.001***).



8.3.3. Chemical composition of fungal sporocarps

There were differences in nutrient concentrations between the fungal species. *Volvopluteus gloiocephalus* fruiting bodies presented significantly higher contents of N, P, K and Mg compared to *Laccaria laccata* sporocarps, whilst this tendency was the opposite for Ca, with higher concentrations in *Laccaria laccata* fruiting bodies. There was no significant difference between fungal species regarding C content (Fig. 8.2).

The two fungal species had different patterns of accumulation for most of the trace elements (Fig. 8.3), with higher concentrations of As, Co, Cu, Fe, Ni, Pb and Zn in *Laccaria laccata* fruiting bodies, and of Cd and S in *Volvopluteus gloiocephalus* fruiting bodies. No significant differences were found for Mn among fungal sporocarps of these species.

Figure 8.2 Nutrients in *Laccaria laccata* and *Volvopluteus gloiocephalus* sporocarps. Bars represent mean values and error bars represent standard error for N = 10. *P* values resulting from Student's *t*-test are indicated (ns: non-significant; $p < 0.01$**; $p < 0.001$***).

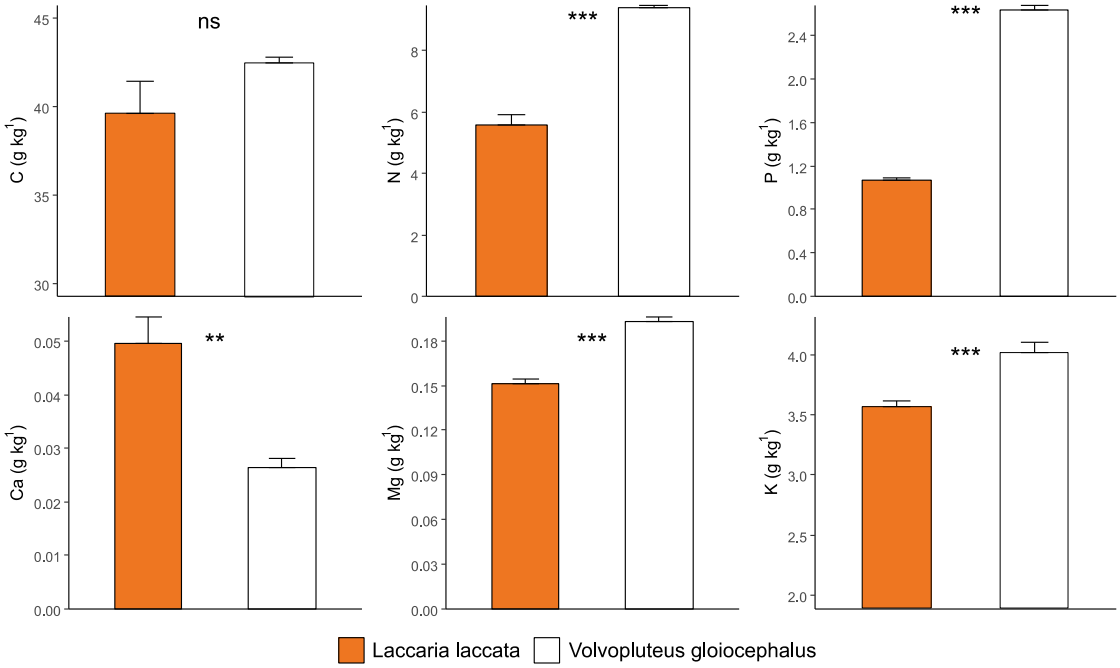
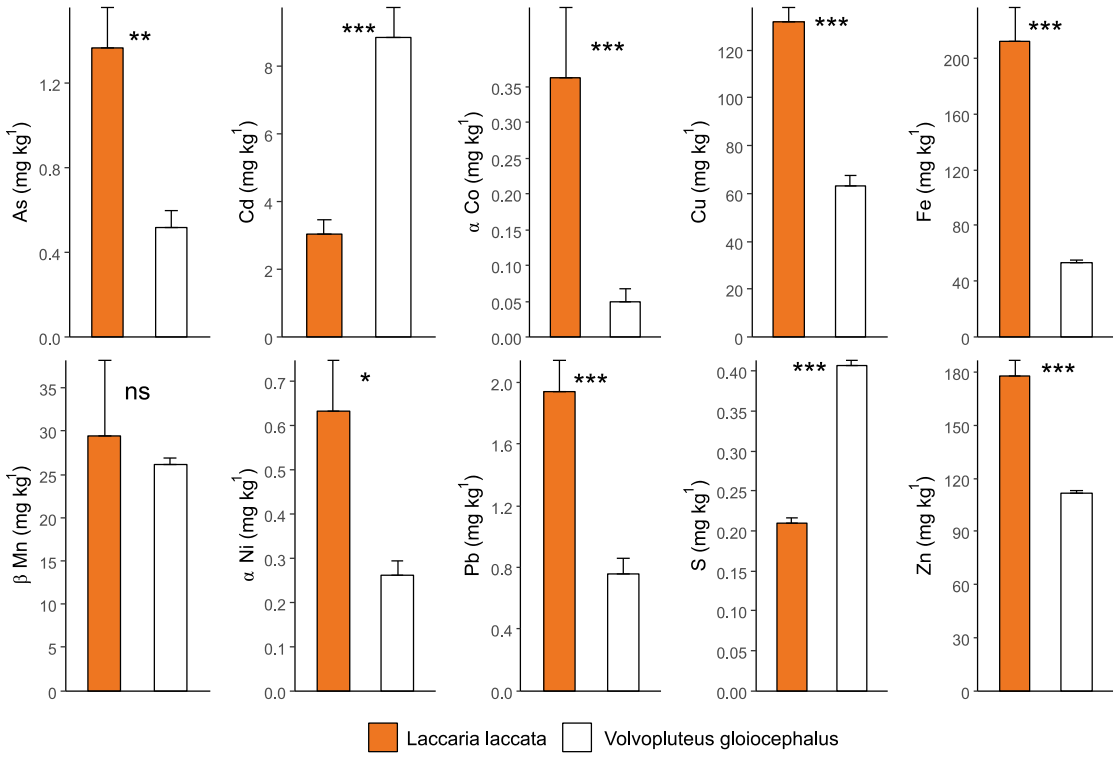


Figure 8.3 Trace elements in *Laccaria laccata* and *Volvopluteus gloiocephalus* sporocarps. Bars represent mean values and error bars represent standard error for N = 10. Student's *t*-test statistic and *p* value are indicated (ns: non-significant; $p < 0.05$*; $p < 0.01$**; $p < 0.001$***). [α] Data transformation for normality of the residuals. [β] Non-parametric Mann-Whitney test (U statistic).



8.3.4. Bioconcentration factors of trace elements

Cadmium BCF was high in both fungal species (means of 5.09 - 8.49; Table 8.3). Mean BCF values of Co, Cu and Zn were close to one (and maximum values exceeded the unity) in the mycorrhizal *Laccaria laccata*, but not in the saprotrophic *Volvopluteus gloiocephalus*. The rest of the trace elements (As, Fe, Mn, Ni, Pb and S) had low BCF values for both species.

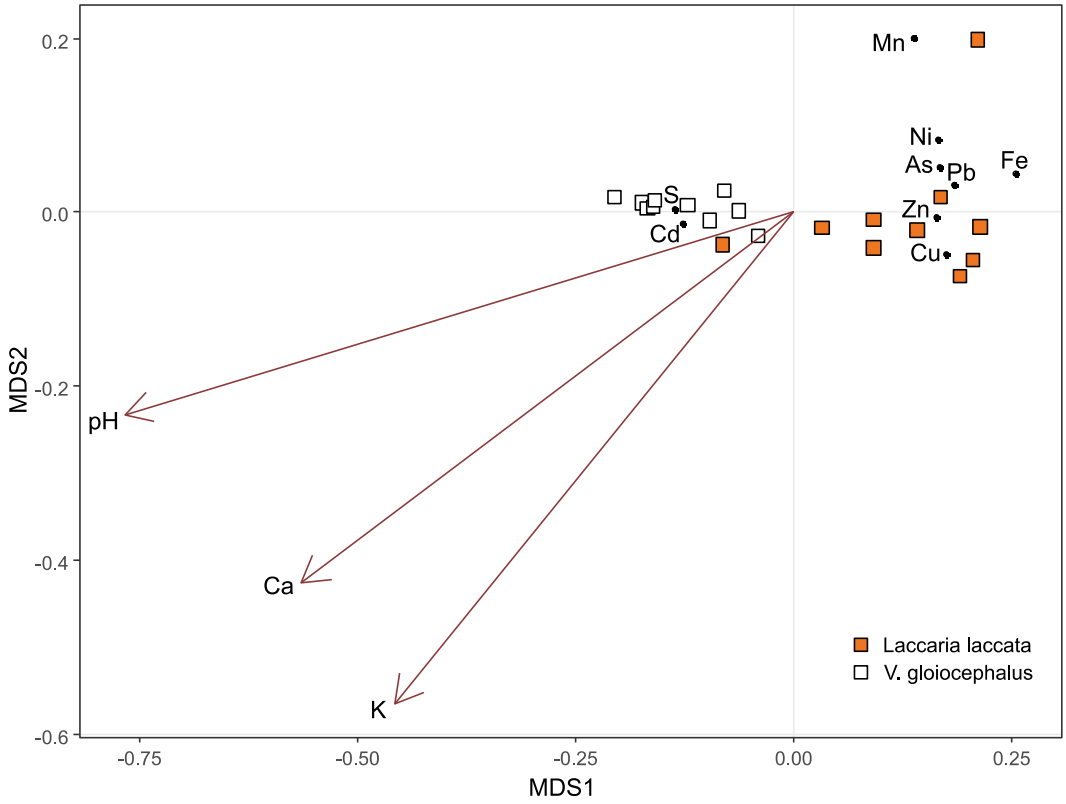
Table 8.3 Bioconcentration factor (BCF) of pseudo-total trace elements in *Laccaria laccata* (*L*) and *Volvopluteus gloiocephalus* (*V*). Bold values indicate BCF > 1.

BCF	Species	Mean	SD	Min	Max
As	*L*	0.0096	0.0040	0.002	0.015
	V	0.0044	0.0021	0.002	0.009
Cd	*L*	**5.09**	**2.87**	**2.75**	**12.58**
	V	**8.49**	**3.34**	**4.98**	**13.66**
Co	*L*	0.626	0.602	0.204	**2.286**
	V	0.034	0.042	0.001	0.138
Cu	*L*	0.795	0.217	0.462	**1.186**
	V	0.351	0.114	0.243	0.634
Fe	*L*	0.0057	0.0018	0.031	0.0079
	V	0.0015	0.0003	0.001	0.0021
Mn	*L*	0.107	0.119	0.049	0.442
	V	0.064	0.011	0.040	0.077
Ni	*L*	0.044	0.027	0.018	0.100
	V	0.018	0.007	0.006	0.028
Pb	*L*	0.0086	0.0021	0.006	0.012
	V	0.0040	0.0027	0.002	0.011
S	*L*	0.0001	<0.0001	0.0001	0.0002
	V	0.0003	0.0002	0.0001	0.0006
Zn	*L*	0.942	0.173	0.715	**1.296**
	V	0.432	0.066	0.351	0.561

According to the NMDS ordination, the soil variables that best explained BCF variability were pH, total Ca and available K (Fig. 8.4). *Volvopluteus gloiocephalus* sporocarps fructified in soils with higher pH and higher Ca and K concentrations compared to *Laccaria laccata* sporocarps. Two defined clusters separated the fungal species with a

higher BCF of Cd and S in *Volvopluteus gloiocephalus*, while *Laccaria laccata* sporocarps presented higher BCF for the rest of the elements (Fig. 8.4).

Figure 8.4 Nonmetric multidimensional scaling (NMDS) ordination of trace element bioconcentration factors (BCF) of *Laccaria laccata* (orange squares) and *Volvopluteus gloiocephalus* (grey squares) fungal species. Black dots represent BCF for each trace element. Brown arrows represent soil variables with a significant correlation with BCF (p = 0.001 for pH and K, p = 0.003 for Ca).



The transfer ratio of trace elements from soil ($CaCl_2$-extracted fraction) to fungal sporocarps was very high for Cd and Cu, moderate for Zn and Fe, low for Co, Mn and Ni, and negligible (< 1) for S (Fig. S8.1). The transfer ratios from soil to fungus were higher in *Volvopluteus gloiocephalus*, in particular for Cd (x12), Zn (x6) and Mn (x5), with the exception of Fe which was higher (x2) in *Laccaria laccata*.

In general, Pearson´s correlation coefficients between trace element concentrations in fungal sporocarps and their underlying soils ($CaCl_2$-extracted values) were low (Table S8.1). *Laccaria laccata* had a significant correlation ($p < 0.05$) with Co, and marginally significant correlations ($p < 0.10$) with As and Mn, and *Volvopluteus gloiocephalus* with Fe.

8.3.5. Toxicity risk

Calculations of the minimum daily intake of the studied fruiting bodies to reach maximum tolerable intake limits for toxicity in humans provided weights over 1 kg of fresh weight of both species for As, Cu, Fe, Pb and Zn. However, the consumption of 0.069 kg fresh weight of *Volvopluteus gloiocephalus* or 0.132 kg fresh weight of *Laccaria laccata* would be enough to reach the daily limit for Cd (Table S8.2). (All the calculations were based on a person of 70 kg bodyweight).

8.4. Discussion

Soil microorganisms play a key role in nutrient cycling, plant symbioses, organic matter decomposition, and other ecosystem processes(Nannipieri et al., 2017). Trace element contaminated soils are of concern due to their toxic effects on soil microbes(Giller et al., 1998). In these soils, fungal communities provide many ecosystem services of regulation; in general, improving soil and water quality, nutrient cycling, soil fertility and carbon sequestration (Bakker et al., 2019). In particular, the "mycoremediation" potential to stabilise trace elements in fungal tissues can be considered another ecosystem service. Thus, fungal mycelium secretes extracellular enzymes and acids to break down contaminants and has a high metal-binding capacity playing a promising role in remediation of trace elements(Purohit et al., 2018). However, from fungal mycelium, trace elements could be channelling to fruiting bodies implying a nutritional potential hazard due to mushroom consumption (Purohit et al., 2018). Fruiting bodies (mushrooms) of more than 1100 fungal species are used worldwide for food and medicines, thus delivering provision and cultural services (Boa, 2004). With exception of poisonous species or edible fungi with high concentration of potentially toxic trace elements, which are a hazard for human health, and then represent a cultural "disservice" (Bakker et al., 2019).

The large-scale phytoremediation plan within the contaminated area considered in this study included planting of native trees and shrubs (Madejón et al., 2018). With time, planted trees modified the properties of underlying soil; for example, pine trees induced soil acidification and, consequently, higher concentrations of soluble fractions of trace

elements (Madejón et al., 2018; Marañón et al., 2015). Trees also influenced surrounding soil microbial activities, compared with adjacent treeless patches. In this heterogeneous landscape mosaic the two studied fungal species fructified in contrasting habitats and soils: *Laccaria laccata* was associated with their host pine trees while saprotrophic *Volvopluteus gloiocephalus* was abundant in the grasslands without trees. Soils surrounding *Volvopluteus gloiocephalus* sporocarps presented higher pseudo-total concentrations of some elements (Cd, Co, Mn and Zn) than *Laccaria laccata* soils (under pine trees). These differences may be caused by the acidification process which increases losses of trace elements from soil due to enhanced leaching and take up by vegetation and fauna (Bolan et al., 2014). However, elements with low mobility, such as As and Pb, had similar concentrations between soil types. The opposite pattern was found for $CaCl_2$-extracted trace element values; most of the trace elements were found in higher concentrations in soils surrounding *Laccaria laccata* sporocarps under pine trees probably due to the pine-induced soil acidification and subsequent trace element mobilisation, as already mentioned.

The differences in habitat and in life history strategy influence the mineral nutrition of both fungal species. *Laccaria laccata* sporocarps were found in soils close to their hosts *Pinus pinea* and *Cistus albidus*, with which they develop symbiotic relationships. The lower nutrient concentration of the mycorrhizal sporocarps, compared with those of *Volvopluteus gloiocephalus*, could be explained by the symbiotic association as pine and cistus may receive part of the nutrients taken up by this fungi, such as N and P(Mayor et al., 2009; Talbot et al., 2013). In contrast, the saprotrophic *Volvopluteus gloiocephalus*, found in grassland areas dominated by herbaceous species, completely relies on soil sources for nutrition without loss by transfer to other organisms. The nutritional quality of both fungal sporocarps was within the usual content range for wild growing mushrooms, except for Mg that was lower in both species (Kalač, 2009).

Contrasting life history strategy of the studied fungal species influences C and N cycles. Despite similar soil C content, the observed differences in soil $\delta^{13}C$ values may reflect a different isotope composition or different turnover rates, and the preferential use of ^{12}C compared to ^{13}C in biological processes (Dawson et al., 2002a). Carbon isotope composition in *Volvopluteus gloiocephalus* fruiting bodies matched the ratios of the C soil source, according to its saprotrophic nature as litter decay fungi. In contrast, *Laccaria laccata* fruiting bodies were ^{13}C-depleted (-28.14 ‰) compared to soil (-26.49 ‰), reflecting the isotope composition of its host photosynthate, which could be the main C

source (Hobbie et al., 1999a; Rosling et al., 2004). The differences in N isotope composition between both fungal species were even more conspicuous, where ectomycorrhizal *Laccaria laccata* fruiting bodies were ^{15}N-enriched in comparison to the surrounding soil; potentially because of the transfer of ^{15}N-depleted compounds to the symbiotic plants (Gebauer and Taylor, 1999; Hobbie et al., 2001, 1999a, 1999b; Hou et al., 2012; Kohzu et al., 2000, 1999). Whereas, saprotrophic *Volvopluteus gloiocephalus* fruiting bodies had much lower ^{15}N than *Laccaria laccata*, and were ^{15}N-depleted in comparison to the soil, despite having similar N isotope composition in both soil types.

A previous study found fungi were able to take up and accumulate trace elements such as Cd, Cu and Zn in both sporocarps and mycelium (BCF from 1.9 to 8.8) in respect of bulk soil concentrations(Vinichuk, 2013). Low selectivity of fungal transporters for essential elements, such as Ca and Zn, favoured the transport of toxic ions with similar chemical properties, such as Cd (Baldrian, 2010). In the study area, both *Volvopluteus gloiocephalus* and *Laccaria laccata* sporocarps mainly bioconcentrated Cd. Previous studies in the same area had found that roots of seven tree species also concentrated Cd (BCF > 1), making them suitable for the phytostabilisation of this potentially toxic element (Madejón et al., 2018). Although Cd was accumulated in fruiting bodies of both fungal species, the patterns and relations with soil differed; *Volvopluteus gloiocephalus* sporocarps accumulated much higher levels of Cd than *Laccaria laccata*. However, accumulation of Ca and Zn were opposite, with higher concentrations in *Laccaria laccata* sporocarps. The correlation between Cd levels in fruiting bodies and soils was positive for the saprotrophic species but negative for the ectomycorrhizal one; a possible explanation is that Cd-Zn-Ca competition influence elements accumulation in studied sporocarps (Hartley et al., 1997a). Higher Ca and Zn content was registered in *Laccaria laccata* sporocarps, besides *Volvopluteus gloiocephalus* surrounding soils recorded higher Ca and Zn concentrations. Therefore, a competition between Cd-Zn-Ca could be responsible for the preference of translocation of Ca and Zn over Cd in *Laccaria laccata* fruiting bodies. On the other hand, the presence of Cd could induce the production of intracellular binding compounds such as metallothioneins, cadystin, and phytochelatins, and cellular compartmentalization in this species(Baldrian, 2010; Blaudez et al., 2000; Guerinot, 2000). Besides the antagonism or interaction between chemical elements, the bioconcentration of trace elements in fungal fruiting bodies is influenced by soil properties (Elekes et al., 2010). In this study, soil pH, Ca and K were relevant variables explaining the BCF of trace elements in fruiting bodies, and these variables were

positively correlated among them ($p < 0.001$). In acidic soils, the release of most trace elements increases, being more available in the soil solution and, therefore, more accessible to microorganisms (Kabata-Pendias and Pendias, 2001; Ledin et al., 1999). In comparison with other studies, As concentrations in *Laccaria laccata* sporocarps are broad, depending on the location and soil conditions, with a range from 0.66 to 147 mg kg^{-1} in caps and stipes (Zhang et al., 2015).

The $CaCl_2$-extracted fraction for most of the trace elements in the soil usually represents a small proportion of their total concentration (Table 2). Therefore, the transfer ratios of trace elements from soil ($CaCl_2$-extracted pool) to fungal sporocarps are functionally more realistic than BCF values (calculated using pseudo-total soil concentrations) and worthwhile to consider. Thus, besides the important Cd accumulation already discussed above, we have detected a high transfer of Cu from soil to fungus (over 200 times). Other trace elements with remarkable soil to fungus transfer were Zn (78 times more in *Volvopluteus gloiocephalus*) and Fe (48 times more in *Laccaria laccata*). If we assume that sporocarps are reflecting mycelium chemical composition, we can infer that both fungal species are contributing potentially to the "mycostabilisation" of Cd, Cu, Zn and Fe in these contaminated and remediated soils (Ali et al., 2017; Purohit et al., 2018).

The well-known capacity of mushrooms to accumulate trace elements could make them useful organisms to bioindicate soil contamination (Markert et al., 2003). However, in this study, trace element concentrations in fruiting bodies were not significantly correlated to soil $CaCl_2$-extracted concentrations, with the exception of Co in *Laccaria laccata*. Many studies have also found that fruiting bodies are not correlated with soil contamination by trace elements; therefore concluding that they are not good bioindicators of soil trace element contamination (Cocchi et al., 2006; Kalač, 2010; Melgar et al., 2016; Petrini et al., 2009). On the contrary, there are authors supporting the use of fungal sporocarps as bioindicators (Alonso et al., 2003; Malinowska et al., 2004; Proskura et al., 2017). In the same study site, the concentration of trace elements in leaves of poplar trees and in soil were correlated for Cd, Cu, Mn and Zn (Madejón et al., 2004). The potential utility as bioindicator would then depend on the fungal species, the particular trace element, and the environmental conditions.

From the food quality perspective, the usually high accumulation of potentially toxic elements in fungal fruiting bodies is a relevant ecosystem disservice. Trace elements concentrations in fruiting bodies were similar to those measured in edible mushrooms

from unpolluted areas (Kalač, 2010), except for the increased mean concentrations of Cd in *Volvopluteus gloiocephalus* (and maximum in *Laccaria laccata*) and mean Cu and maximum Fe, Mn and Zn concentrations in *Laccaria laccata* sporocarps (Table S3).

Food regulation to avoid toxicity risk includes concentration limits for several trace elements in wild edible mushrooms. Thus, the European Union regulation established a concentration limit of 0.3 mg Pb kg^{-1} fresh weight and 0.2 mg Cd kg^{-1} fresh weight for cultivated fungal species (*Agaricus bisporus*, *Pleurotus ostreatus* and *Lentinula edodes*). For other fungal species, this limit was established at 1.00 mg Cd kg^{-1} fresh weight and no regulation limit was established in other species for Pb (European Commission, 2015, 2014). The maximum Cd concentration registered in *Volvopluteus gloiocephalus* sporocarps was 0.84 mg Cd kg^{-1} fresh weight, which is well above the European Union regulation limit for cultivated mushrooms, although still below the limit for wild species. The maximum Pb concentration in our sporocarps was 0.25 mg Pb kg^{-1} fresh weight, which is also below the limit (for cultivated species only). Therefore, according to European Union regulation, neither Cd nor Pb concentrations reached these maximum levels (Table S1; dry weight).

The World Health Organization (WHO, 2010) evaluated certain trace element contaminants in food to estimate the tolerable intake limits (Table S2; PMTDI). We calculated the minimum weight to reach tolerable limits, and Cd presented the lowest weight (Table S2). A daily intake of 0.069 kg fresh weight of *Volvopluteus gloiocephalus* or 0.132 kg fresh weight of *Laccaria laccata* (for a person with 70 kg bodyweight) would reach the maximal tolerable Cd daily limit, without taking into account other Cd daily sources. The consumption of these fungi could represent a toxicological risk due to elevated Cd content, especially *Volvopluteus gloiocephalus* species, as this element is of high toxicological importance for humans (Kalač, 2010; Kalač and Svoboda, 2000). *Laccaria laccata* consumption would entail a higher toxicological risk than *Volvopluteus gloiocephalus* for the rest of trace elements (As, Cu, Fe, Pb and Zn), however the daily intake to reach toxicity (over 1 kg fresh weight) represents an unrealistically high level of consumption. In any case, for a complete evaluation of actual potential toxicity to humans the effect of mushroom processing during cooking on its metal concentrations should also be taken into account.

8.5. Conclusions

After a long-term restoration process to mitigate the effects of a mine-spill in SW Spain, *Laccaria laccata* and *Volvopluteus gloiocephalus* sporocarps and surrounding soil analyses demonstrated trace element persistence in the study area. Some soil trace element concentrations were above background values and soil acidification seemed to be responsible for the higher trace element concentrations in *Laccaria laccata* fruiting bodies. Analyses of $\delta^{15}N$ and $\delta^{13}C$ in fruiting bodies and surrounding soils allowed us to differentiate the saprotrophic *Volvopluteus gloiocephalus* fungi from the ectomycorrhizal *Laccaria laccata* fungi, proving this technique as adequate to differentiate fungal life history and to contribute to a better knowledge of the soil-fungi relations.

Bioconcentration factors demonstrated that both fungal species accumulated Cd, and that Cu was transferred from the soil "available" ($CaCl_2$-extracted) pool to fungal sporocarps. Human consumption of *Laccaria laccata* and/ or *Volvopluteus gloiocephalus* sporocarps collected in the area may represent a toxicological risk due to the elevated concentrations of Cd. Therefore, strict control is required in the area to avoid human consumption of these fruiting bodies. Due to the ability of these species to accumulate some metals, monitoring on trace element in these fungi is thus highly recommended, even if mobility of these metals in soils is assumed to be low.

8.6. Bibliography

Agerer, R., Christan, J., Mayr, C., Hobbie, E., 2012. Isotopic signatures and trophic status of Ramaria. Mycological Progress 11, 47–59.

Ali, A., Guo, D., Mahar, A., Wang, P., Shen, F., Li, R., Zhang, Z., 2017. Mycoremediation of potentially toxic trace elements—a biological tool for soil cleanup: a review. Pedosphere 27, 205–222.

Alonso, J., García, M.A., Pérez-López, M., Melgar, M.J., 2003. The concentrations and bioconcentration factors of copper and zinc in edible mushrooms. Archives of Environmental Contamination and Toxicology 44, 180–188.

Bakker, M.R., Brunner, I., Ashwood, F., Bjarnadottir, B., Bolger, T., Børja, I., Carnol, M., Cudlin, P., Dalsgaard, L., Erktan, A., Godbold, D., Kraigher, H., Meier, I.C., Merino-Martín, L., Motiejūnaitė, J., Mrak, T., Oddsdóttir, E.S., Ostonen, I., Pennanen, T.L., Püttsepp, Ü., Suz, L.M., Vanguelova, E.I., Vesterdal, L., Soudzilovskaia, N.A., 2019. Belowground biodiversity relates positively to ecosystem services of European forests. Frontiers in Forests and Global Change 2, 1–23.

Baldrian, P., 2010. Effect of heavy metals on saprotrophic soil fungi, in: Sherameti, I., Varma, A. (Eds.), Soil Heavy Metals. Springer-Verlag, Berlin Heidelberg, pp. 263–279.

Bellion, M., Courbot, M., Jacob, C., Blaudez, D., Chalot, M., 2006. Extracellular and cellular mechanisms sustaining metal tolerance in ectomycorrhizal fungi. FEMS Microbiology Letters 254, 173–181.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate : a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society. Series B (Methodological) 57, 289–300.

Blaudez, D., Botton, B., Chalot, M., 2000. Cadmium uptake and subcellular compartmentation in the ectomycorrhizal fungus Paxillus involutus. Microbiology 146, 1109–1117.

Boa, E., 2004. Wild Edible Fungi: A global overview of their use and importance to people, in: Non-wood forest products. Food and Agriculture Organization of the United Nations, Rome.

Bolan, N., Kunhikrishnan, A., Thangarajan, R., Kumpiene, J., Park, J., Makino, T., Kirkham, M.B., Scheckel, K., 2014. Remediation of heavy metal(loid)s contaminated soils - to mobilize or to immobilize? Journal of Hazardous Materials 266, 141–166.

Buscaroli, A., 2017. An overview of indexes to evaluate terrestrial plants for phytoremediation purposes (Review). Ecological Indicators 82, 367–380.

Cocchi, L., Vescovi, L., Petrini, L.E., Petrini, O., 2006. Heavy metals in edible mushrooms in Italy. Food Chemistry 98, 277–284.

Colpaert, J. V., Van Assche, J.A., 1992. The effects of cadmium and the cadmium-zinc interaction on the axenic growth of ectomycorrhizal fungi. Plant and Soil 145, 237–243.

Colpaert, J. V., Wevers, J.H.L., Krznaric, E., Adriaensen, K., 2011. How metal-tolerant ecotypes of ectomycorrhizal fungi protect plants from heavy metal pollution. Annals of Forest Science 68, 17–24.

Courty, P.E., Bréda, N., Garbaye, J., 2007. Relation between oak tree phenology and the secretion of organic matter degrading enzymes by *Lactarius quietus* ectomycorrhizas before and during bud break. Soil Biology and Biochemistry 39, 1655–1663.

Courty, P.E., Buée, M., Diedhiou, A.G., Frey-Klett, P., Le Tacon, F., Rineau, F., Turpault, M.P., Uroz, S., Garbaye, J., 2010. The role of ectomycorrhizal communities in forest ecosystem processes: new perspectives and emerging concepts. Soil Biology and Biochemistry 42, 679–698.

Dawson, T.E., Mambelli, S., Plamboeck, A.H., Templer, P.H., Tu, K.P., 2002. Stable isotopes in plant ecology. Annual Review of Ecology and Systematics 33, 507–559.

Dix, N.J., Webster, J., 1995. Terrestrial Macrofungi, in: Fungal Ecology. Springer Netherlands, Dordrecht, pp. 341–397.

Domínguez, M.T., Alegre, J.M., Madejón, P., Madejón, E., Burgos, P., Cabrera, F., Marañón, T., Murillo, J.M., 2016. River banks and channels as hotspots of soil pollution after large-scale remediation of a river basin. Geoderma 261, 133–140.

Elekes, C.C., Busuioc, G., Ionita, G., 2010. The bioaccumulation of some heavy metals in the fruiting body of wild growing mushrooms. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 38, 147–151.

European Commission, 2015. Commission Regulation (EU) 2015/1005 of 25 June 2015 amending Regulation (EC) No 1881/2006 as regards maximum levels of lead in certain foodstuffs, Official Journal of the European Union.

European Commission, 2014. Commission Regulation (EU) No 488/2014 of 12 May 2014 amending Regulation (EC) No 1881/2006 as regards maximum levels of cadmium in foodstuffs, Official Journal of the European Union.

European Committee for Standardization, 2008. UNE-EN 15517:2008. Foodstuffs - Determination of trace elements - Determination of inorganic arsenic in seaweed by hydride generation atomic absorption spectrometry (HGAAS) after acid extraction.

Falandysz, J., Saba, M., Liu, H., Li, T., Wang, J., Wiejak, A., Zhang, J., Wang, Y., Zhang, D., 2016. Mercury in forest mushrooms and topsoil from the Yunnan highlands and the subalpine region of the Minya Konka summit in the Eastern Tibetan Plateau. Environmental Science and Pollution Research 23730–23741.

Galán, E., Romero, A., 2008. Contaminación de suelos por metales pesados, in: Sociedad Española de Mineralogía. pp. 48–60.

Gastwirth, J.L., Gel, Y.R., Hui, W.L.W., Lyubchich, V., Miao, W., Noguchi, K., 2019. Lawstat: tools for biostatistics, public policy, and law.

Gebauer, G., Taylor, A.F.S., 1999. 15N natural abundance in fruit bodies of different functional groups of fungi in relation to substrate utilization. New Phytologist 143, 93–101.

Giller, K.E., Witter, E., Mcgrath, S.P., 1998. Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. Soil Biology and Biochemistry 30, 1389–1414.

Godbold, D.L., Jentschke, G., Winter, S., Marschner, P., 1998. Ectomycorrhizas and amelioration of metal stress in forest trees. Chemosphere 36, 757–762.

Gross, J., Ligges, U., 2015. Nortest: tests for normality, Version 1.0-4.

Guerinot, M. Lou, 2000. The ZIP family of metal transporters. Biochimica et Biophysica Acta 1465, 190–198.

Hartley, J., Cairney, J.W.G., Meharg, A.A., 1997a. Do ectomycorrhizal fungi exhibit adaptive tolerance to potentially toxic metals in the environment? Plant and Soil 189, 303–319.

Hartley, J., Cairney, J.W.G., Sanders, F.E., Meharg, A.A., 1997b. Toxic interactions of metal ions (Cd^{2+}, Pb^{2+}, Zn^{2+} and Sb^{3-}) on in vitro biomass production of ectomycorrhizal fungi. New Phytologist 137, 551–562.

Hobbie, E., Högberg, P., 2012. Nitrogen isotopes link mycorrhizal fungi and plants to nitrogen dynamics. New Phytologist 196, 367–382.

Hobbie, E.A., Horton, T.R., 2007. Evidence that saprotrophic fungi mobilise carbon and mycorrhizal fungi mobilise nitrogen during litter decomposition. New Phytologist 173, 447–449.

Hobbie, E.A., Macko, S.A., Shugart, H.H., 1999a. Insights into nitrogen and carbon dynamics of ectomycorrhizal and saprotrophic fungi from isotopic evidence. Oecologia 118, 353.

Hobbie, E.A., Macko, S.A., Shugart, H.H., 1999b. Interpretation of nitrogen isotope signatures using the NIFTE model. Oecologia 120, 405–415.

Hobbie, E.A., Sánchez, F.S., Rygiewicz, P.T., 2004. Carbon use, nitrogen use, and isotopic fractionation of ectomycorrhizal and saprotrophic fungi in natural abundance and 13C-labelled cultures. Mycological Research 108, 725–736.

Hobbie, E.A., Weber, N.S., Trappe, J.M., 2001. Mycorrhizal vs saprotrophic status of fungi: the isotopic evidence. New Phytologist 150, 601–610.

Hou, W., Lian, B., Dong, H., Jiang, H., Wu, X., 2012. Distinguishing ectomycorrhizal and saprophytic fungi using carbon and nitrogen isotopic compositions. Geoscience Frontiers 3, 351–356.

Houba, V.J.G., Temminghoff, E.J.M., Gaikhorst, G.A., van Vark, W., 2000. Soil analysis procedures using 0.01 M calcium chloride as extraction reagent. Communications in Soil Science and Plant Analysis 31, 1299–1396.

Ishida, T.A., Nara, K., Tanaka, M., Kinoshita, A., Hogetsu, T., 2008. Germination and infectivity of ectomycorrhizal fungal spores in relation to their ecological traits during primary succession. New Phytologist 180, 491–500.

Justo, A., Minnis, A.M., Ghignone, S., Menolli, N., Capelari, M., Rodríguez, O., Malysheva, E., Contu, M., Vizzini, A., 2011. Species recognition in *Pluteus* and *Volvopluteus* (Pluteaceae, Agaricales): morphology, geography and phylogeny. Mycological Progress 10, 453–479.

Kabata-Pendias, A., Pendias, H., 2001. Trace elements in soils and plants, 3rd ed. CRC Press LLC, Boca Raton.

Kalač, P., 2010. Trace element contents in European species of wild growing edible mushrooms: a review for the period 2000-2009. Food Chemistry 122, 2–15.

Kalač, P., 2009. Chemical composition and nutritional value of European species of wild growing mushrooms: a review. Food Chemistry 113, 9–16.

Kalač, P., Svoboda, L., 2000. A review of trace element concentrations in edible mushrooms. Food Chemistry 69, 273–281.

Kohzu, A., Tateishi, T., Yamada, A., Koba, K., Wada, E., 2000. Nitrogen isotope fractionation during nitrogen transport from ectomycorrhizal fungi, *Suillus granulatus*, to the host plant, *Pinus densiflora*. Soil Science and Plant Nutrition 46, 733–739.

Kohzu, A., Yoshioka, T., Ando, T., Takahashi, M., Koba, K., Wada, E., 1999. Natural ^{13}C and ^{15}N abundance of field-collected fungi and their ecological implications. New Phytologist 144, 323–330.

Koide, R.T., Sharda, J.N., Herr, J.R., Malcolm, G.M., 2008. Ectomycorrhizal fungi and the biotrophy–saprotrophy continuum. New Phytologist 178, 230–233.

Komárek, M., Chrastný, V., Štíchová, J., 2007. Metal/metalloid contamination and isotopic composition of lead in edible mushrooms and forest soils originating from a smelting area. Environment International 33, 677–684.

Krpata, D., Fitz, W., Peintner, U., Langer, I., Schweiger, P., 2009. Bioconcentration of zinc and cadmium in ectomycorrhizal fungi and associated aspen trees as affected by level of pollution. Environmental Pollution 157, 280–286.

Kruskal, J.B., 1964. Multidimensional scaling by optimizing goodness of fit to a nonmetric hypothesis. Psychometrika 29, 1–27.

Kuo, S., 1996. Phosphorus, in: Sparks, D.L. (Ed.), Methods of Soil Analysis: Part 3- Chemical Methods. SSSA, Madison, USA, pp. 869–919.

Ledin, M., Krantz-Rülcker, C., Allard, B., 1999. Microorganisms as metal sorbents: comparison with other soil constituents in multi-compartment systems. Soil Biology and Biochemistry 31, 1639–1648.

Lindahl, B.D., Ihrmark, K., Boberg, J., Trumbore, S.E., Högberg, P., Stenlid, J., Finlay, R.D., 2007. Spatial separation of litter decomposition and mycorrhizal nitrogen uptake in a boreal forest. New Phytologist 173, 611–620.

Lindahl, B.D., Tunlid, A., 2015. Ectomycorrhizal fungi - potential organic matter decomposers, yet not saprotrophs. New Phytologist 205, 1443–1447.

Madejón, P., Domínguez, M.T., Gil-Martínez, M., Navarro-Fernández, C.M., Montiel-Rozas, M.M., Madejón, E., Murillo, J.M., Cabrera, F., Marañón, T., 2018. Evaluation of amendment addition and tree planting as measures to remediate contaminated soils: the Guadiamar case study (SW Spain). Catena 166, 34–43.

Madejón, P., Marañón, T., Murillo, J.M., Robinson, B., 2004. White poplar (*Populus alba*) as a biomonitor of trace elements in contaminated riparian forests. Environmental Pollution 132, 145–155.

Malinowska, E., Szefer, P., Falandysz, J., 2004. Metals bioaccumulation by bay bolete, *Xerocomus badius*, from selected sites in Poland. Food Chemistry 84, 405–416.

Marañón, T., Navarro-Fernández, C.M., Domínguez, M.T., Madejón, P., Murillo, J.M., 2015. How the soil chemical composition is affected by seven tree species planted at a contaminated and remediated site. Web Ecology 15, 45–48.

Markert, B.A., Breure, A.M., Zechmeister, H.G., 2003. Trace metals and other contaminants in the environment. Volume 6 - Bioindicators and biomonitors, 1st ed. Pergamon.

Mayor, J.R., Schuur, E.A.G., Henkel, T.W., 2009. Elucidating the nutritional dynamics of fungi using stable isotopes. Ecology Letters 12, 171–183.

McGuire, K.L., Allison, S.D., Fierer, N., Treseder, K.K., 2013. Ectomycorrhizal-dominated boreal and tropical forests have distinct fungal communities, but analogous spatial patterns across soil horizons. PLoS ONE 8, 1–9.

Melgar, M.J., Alonso, J., García, M.A., 2016. Cadmium in edible mushrooms from NW Spain: bioconcentration factors and consumer health implications. Food and Chemical Toxicology 88, 13–20.

Mleczek, M., Siwulski, M., Stuper-Szablewska, K., Rissmann, I., Sobieralski, K., Goliński, P., 2013. Accumulation of elements by edible mushroom species: Part I. Problem of trace element toxicity in mushrooms. Journal of Environmental Science and Health - Part B Pesticides, Food Contaminants, and Agricultural Wastes 48, 69–81.

Nannipieri, P., Ascher, J., Ceccherini, M.T., Landi, L., Pietramellara, G., Renella, G., 2017. Microbial diversity and soil functions. European Journal of Soil Science 68, 12–26.

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., 2019. Vegan: Community Ecology Package, R package version 2.5-6.

Petrini, O., Cocchi, L., Vescovi, L., Petrini, L., 2009. Chemical elements in mushrooms: their potential taxonomic significance. Mycological Progress 8, 171–180.

Phillips, R.P., Brzostek, E., Midgley, M.G., 2013. The mycorrhizal-associated nutrient economy: a new framework for predicting carbon-nutrient couplings in temperate forests. New Phytologist 199, 41–51.

Proskura, N., Skopicz-Radkiewicz, L., Podlasińska, J., 2017. Chemical composition and bioaccumulation ability of *Boletus badius* (Fr.) collected in western Poland. Chemosphere 168, 106–111.

Purohit, J., Chattopadhyay, A., Biswas, M.K., Singh, N.K., 2018. Mycoremediation of agricultural soil: bioprospection for sustainable development, in: Prasad, R. (Ed.), Mycoremediation and Environmental Sustainability. Springer International Publishing AG, Cham, Switzerland, pp. 39–60.

R Core Team, 2018. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Read, D.J., Leake, J.R., Perez-Moreno, J., 2004. Mycorrhizal fungi as drivers of ecosystem processes in heathland and boreal forest biomes. Canadian Journal of Botany 83, 1243–1263.

Revelle, W., 2019. Psych: procedures for psychological, psychometric, and personality research, R package version 1.9.12.

Rosling, A., Lindahl, B.D., Finlay, R.D., 2004. Carbon allocation to ectomycorrhizal roots and mycelium colonising different mineral substrates. New Phytologist 162, 795–802.

Sinsabaugh, R.L., Lauber, C.L., Weintraub, M.N., Ahmed, B., Allison, S.D., Crenshaw, C., Contosta, A.R., Cusack, D., Frey, S., Gallo, M.E., Gartner, T.B., Hobbie, S.E., Holland, K., Keeler, B.L., Powers, J.S., Stursova, M., Takacs-Vesbach, C., Waldrop, M.P.,

Wallenstein, M.D., Zak, D.R., Zeglin, L.H., 2008. Stoichiometry of soil enzyme activity at global scale. Ecology Letters 11, 1252–1264.

Smith, S., Read, D., 2008. Mycorrhizal Symbiosis, 3rd ed. Academic Press, London.

Svoboda, L., Havlíčková, B., Kalač, P., 2006. Contents of cadmium, mercury and lead in edible mushrooms growing in a historical silver-mining area. Food Chemistry 96, 580–585.

Talbot, J.M., Bruns, T.D., Smith, D.P., Branco, S., Glassman, S.I., Erlandson, S., Vilgalys, R., Peay, K.G., 2013. Independent roles of ectomycorrhizal and saprotrophic communities in soil organic matter decomposition. Soil Biology and Biochemistry 57, 282–291.

Tyler, G., 1982. Metal accumulation by wood-decaying fungi. Chemosphere 11, 1141–1146.

Venables, W.N., Ripley, B.D., 2002. Modern Applied Statistics with S, 4th ed. Springer, New York.

Vinichuk, M.M., 2013. Copper, zinc, and cadmium in various fractions of soil and fungi in a Swedish forest. Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering 48, 980–987.

WHO, 2017. Evaluations of the Joint FAO/WHO Expert Committee on Food Additives (JECFA). 84th JECFA.

WHO, 2010. Summary Report of the 72nd Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

Wickham, H., 2016. ggplot2: elegant graphics for data analysis. Springer-Verlag, New York.

Zhang, J., Li, T., Yang, Y.L., Liu, H.G., Wang, Y.Z., 2015. Arsenic concentrations and associated health risks in *Laccaria* mushrooms from Yunnan (SW China). Biological Trace Element Research 164, 261–266.

8.7 Supplementary material

Figure S8.1 Transfer ratio (in logarithmic scale) of seven trace elements and S from soil ($CaCl_2$-extractable pool) to fungal sporocarps for the two study species: *Laccaria laccata* (orange bars) and *Volvopluteus goiocephalus* (white bars).

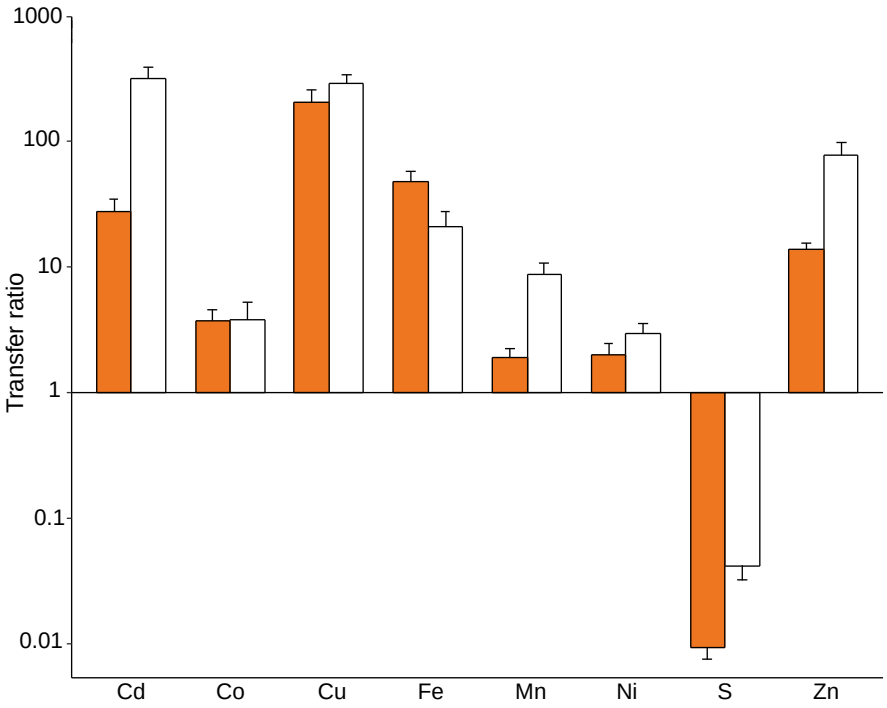


Figure S8.2 Location map of the studied area within the Guadiamar Green Corridor (SW Spain) with sampled points of *Laccaria laccata* (orange squares) and *Volvopluteus gloiocephalus* (white squares). Map image form Google Earth, version 7.3.2.5776 (earth.google.com/web/).

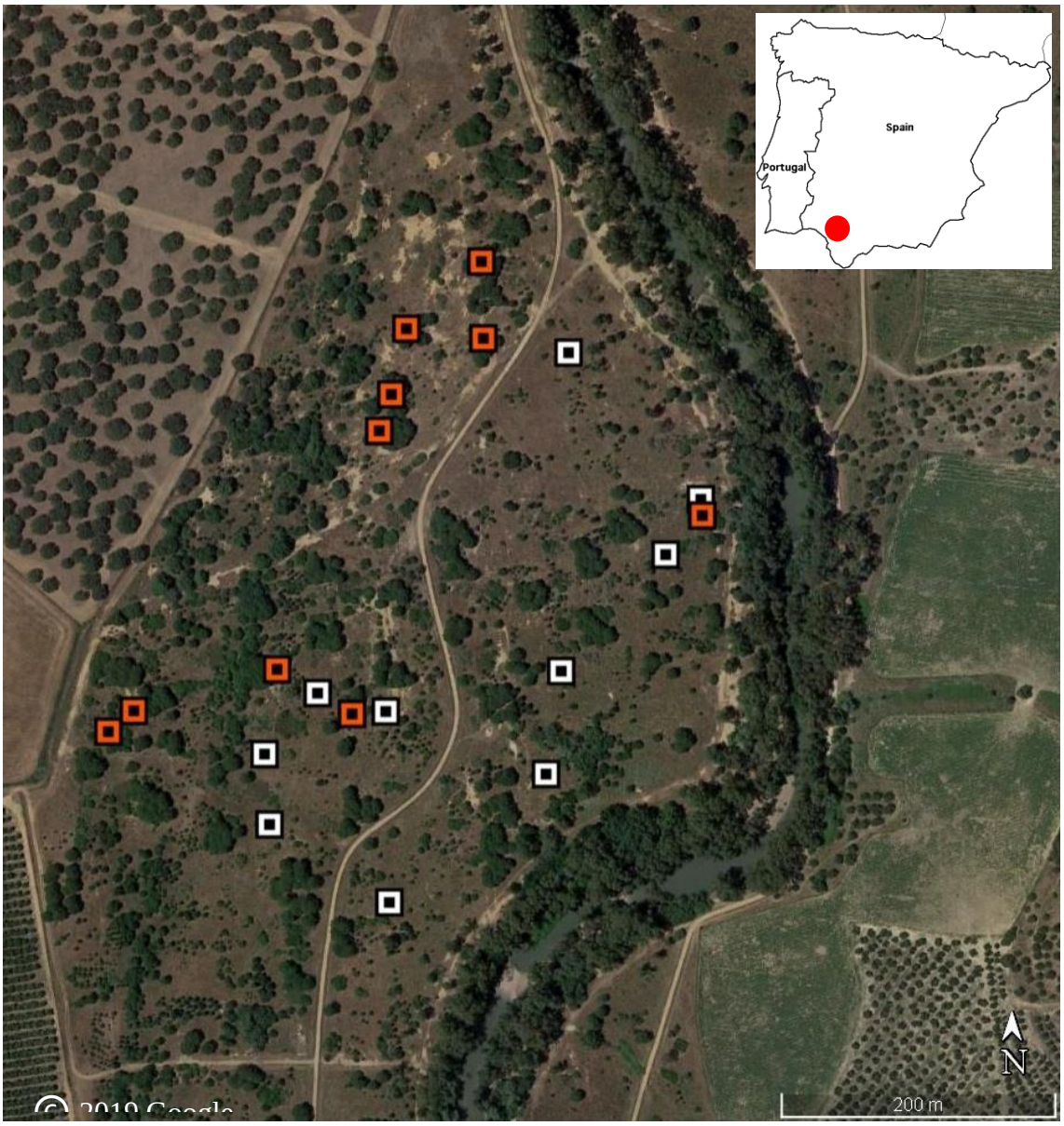


Table S8.1 Correlations between trace elements and S in *Laccaria laccata* and *Volvopluteus gloiocephalus* sporocarps and their corresponding underneath soils ($CaCl_2$-extractable concentrations, except pseudo-total As and Pb). Coefficient of Pearson correlations (*r*) and probability value (*p*) are indicated for N = 10. Bold values indicate $p < 0.05$.

	Laccaria laccata		***Volvopluteus gloiocephalus***	
	r	*p*	*r*	*p*
As	0.56	0.09	0.42	0.23
Cd	-0.21	0.55	0.38	0.28
Co	**0.79**	**0.01**	0.30	0.40
Cu	-0.30	0.39	-0.47	0.17
Fe	-0.13	0.72	0.60	0.07
Mn	0.57	0.08	0.07	0.85
Ni	-0.04	0.91	-0.05	0.89
Pb	0.50	0.14	0.22	0.55
S	0.19	0.59	-0.31	0.38
Zn	0.25	0.49	-0.25	0.48

Table S8.2 Contaminant provisional daily tolerable intake limits established by the Joint FAO/WHO Expert Committee on Food Additives (WHO, 2017) and minimum fungi daily intake of studied fruiting bodies to reach limits, expressed as daily kg of fungi fw (fresh weight) based on a person of 70 kg bodyweight. PMTDI (provisional maximum tolerable daily intake).

Contaminant	PMTDI ($\mu g\ kg^{-1}$ bw)	*Laccaria laccata* Daily limit (kg fw)	*Volvopluteus gloiocephalus* Daily limit (kg fw)
As	3*	1.29	3.80
Cd	0.83	0.132	0.069
Cu	500	3.19	6.49
Fe	800	2.25	13.52
Pb	3.6*	0.99	3.25
Zn	300-1000	1.30	2.96

*: Revoked limit. Not possible to establish a new intake that would be considered health protective.

Table S8.3 Concentration of trace elements and S (in mg kg^{-1} dry weight) in fruiting bodies of *Laccaria laccata* and *Volvopluteus gloiocephalus* fungal species. Mean, SE and range values for N = 10. For comparison, the usual range values for edible mushrooms from unpolluted sites are indicated (in mg kg^{-1} dry weight) (Kalač, 2010) (n.a. = data non-available). The maximum allowed concentrations for Cd and Pb in wild edible mushrooms for human consumption (European Commission, 2015, 2014) have been converted to mg kg^{-1} dry weight.

Trace element	***Laccaria laccata***				***Volvopluteus gloiocephalus***				**Usual range from unpolluted sites**	**Maximum limit by EU regulation**
	Mean	**SE**	**Min**	**Max**	**Mean**	**SE**	**Min**	**Max**		
As	1.36	0.19	0.26	2.25	0.518	0.075	0.203	1.035	0.5 – 5.0	
Cd	3.02	0.45	1.45	6.01	8.86	0.88	4.82	12.67	1.0 – 5.0	14.0 – 17.3
Co	0.363	0.098	0.134	1.202	0.035	0.015	0.008	0.154	< 0.5	
Cu	132	6	105	159	63.1	4.4	53.7	98.4	20 - 100	
Fe	212	25	116	344	53.0	2.0	44.4	62.5	50 - 300	
Mn	29.4	8.7	15.1	106.1	26.1	0.8	21.2	30.0	10 - 60	
Ni	0.631	0.114	0.289	1.370	0.260	0.033	0.087	0.395	Traces – 15.0	
Pb	1.94	0.20	1.13	3.48	0.757	0.103	0.346	1.404	< 5.0	4.2 – 5.2
S	0.210	0.006	0.176	0.235	0.407	0.006	0.385	0.433	n.a.	
Zn	178	9	129	219	111	2	104	125	25 - 200	

Bibliography

European Commission, 2015. Commission Regulation (EU) 2015/1005 of 25 June 2015 amending Regulation (EC) No 1881/2006 as regards maximum levels of lead in certain foodstuffs, Official Journal of the European Union.

European Commission, 2014. Commission Regulation (EU) No 488/2014 of 12 May 2014 amending Regulation (EC) No 1881/2006 as regards maximum levels of cadmium in foodstuffs, Official Journal of the European Union.

Kalač, P., 2010. Trace element contents in European species of wild growing edible mushrooms: A review for the period 2000-2009. Food Chemistry 122, 2–15. doi:10.1016/j.foodchem.2010.02.045

WHO, 2017. Evaluations of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) [WWW Document]. 84th JECFA. URL http://apps.who.int/food-additives-contaminants-jecfa-database/search.aspx (accessed 4.9.19).

9. DISCUSIÓN GENERAL

En una zona contaminada por elementos traza, objeto de esta Tesis Doctoral, se llevó a cabo una estrategia de fitorremediación para recuperar la funcionalidad del ecosistema después de un accidente minero. Se aplicaron sucesivas fases que fueron la remoción de los lodos mineros, la aplicación de enmiendas y la plantación de diferentes especies leñosas. Desde entonces este ecosistema terrestre novel -el Corredor Verde del Guadiamar- se ha ido desarrollado de manera heterogénea según el tipo de suelo, los niveles de contaminación y las especies plantadas.

El suelo tiene numerosas funciones que son esenciales tanto para el establecimiento y supervivencia de las plantas como para el funcionamiento de los ciclos y procesos del ecosistema terrestre (Bardgett and van der Putten, 2014). Existe una estrecha interacción entre la planta y el suelo que es interesante investigar para conocer qué funciones del ecosistema se están recuperando tras la perturbación y las medidas de remediación acometidas (Krumins et al., 2015). Mediante el estudio del funcionamiento de las comunidades microbianas se puede evaluar de una manera más integrada el proceso desarrollado por la fitorremediación y su impacto en el suelo (Burns et al., 2013; Wang et al., 2007). La microbiota del suelo es un indicador potencial tanto de la perturbación como de la restauración en los ecosistemas terrestres (Dick and Tabatabai, 1992).

Entre las comunidades microbianas del suelo, los hongos son especialmente importantes en las zonas donde se ha establecido una estrategia de fitorremediación, debido a las asociaciones simbióticas que tienen con las plantas (Op De Beeck et al., 2015; van der Heijden et al., 1998). Por su parte, las distintas especies vegetales tienen un impacto específico sobre el suelo (*footprint*), diferente según sus rasgos, su composición química y su estilo de vida, además, de sus interacciones con hongos micorrícicos.

Esta Tesis Doctoral se ha centrado en investigar cómo los diferentes tipos de coberturas vegetales conllevan diferentes relaciones con las comunidades de hongos del suelo en una zona contaminada por elementos traza, y qué efectos tienen estas relaciones tanto en el estado de la planta como en una serie de procesos que participan en los ciclos biogeoquímicos del suelo. La importancia del estudio de las relaciones planta-hongo en ecosistemas en proceso de restauración está motivada por el papel que estos microorganismos realizan en la nutrición y protección de la planta (van der Heijden et al., 2006). En la interacción planta-hongo micorrícico se producen intercambios vitales para ambos; además, en el caso de zonas contaminadas por elementos traza, el hongo puede reducir los efectos negativos de la contaminación en la planta a través de mecanismos de

reducción y movilización de dichos elementos (Cabral et al., 2015; Khan, 2005). Conocer el efecto de estos hongos en la translocación de metales y en el estado nutricional de la planta nos enseña cómo el papel de los hongos es fundamental en el establecimiento y desarrollo de la cubierta vegetal en ecosistemas emergentes.

9.1 Discusión por capítulos de estudio

A continuación, se discuten los principales resultados de la Tesis Doctoral (ver esquema en Fig. 9.1). Los estudios desarrollados en esta Tesis Doctoral han abordado la relación de la actividad microbiana del suelo con la cubierta vegetal (**Capítulo 4**); la diversidad de hongos del suelo y sus principales tipos funcionales (micorrícicos y saprofitos) en relación a las propiedades abióticas de los suelos y el tipo de cubierta vegetal (**Capítulo 5**); los factores que determinan la composición de las comunidades de hongos ectomicorrícicos (ECM) asociados a la encina (**Capítulo 6**) y las interacciones suelo-hongos ECM-encina (**Capítulo 7**); y, por último, la nutrición de los cuerpos fructíferos (setas) y la posible toxicidad para el consumo humano (**Capítulo 8**).

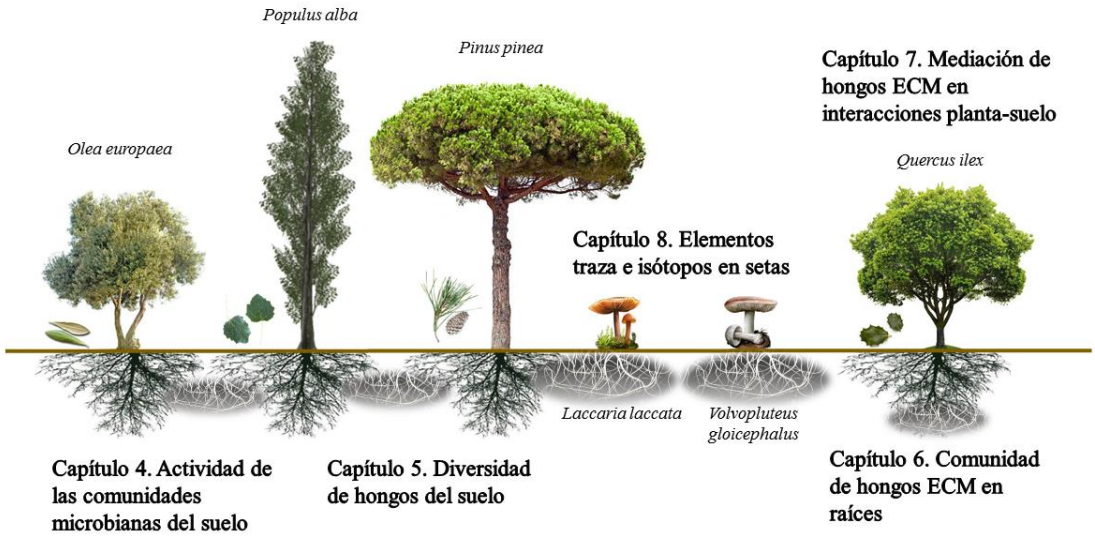


Figura 9.1 Diferentes aspectos estudiados por capítulo en la Tesis Doctoral sobre la fitorremediación y las relaciones planta-hongo-suelo.

En el **Capítulo 4** hemos estudiado los efectos de la fitorremediación en las comunidades microbianas de los suelos contaminados del Corredor Verde del Guadiamar.

En este estudio hemos observado cómo las medidas aplicadas en la fitorremediación han promovido cambios positivos en el medio abiótico y han mejorado la abundancia, la actividad y la funcionalidad de las comunidades microbianas. Estos resultados concuerdan con los de estudios previos realizados en la zona, que muestran que ciertas intervenciones en suelos altamente degradados, como la aplicación de enmiendas para mejorar las condiciones de pH, pueden facilitar la recuperación de las funciones microbianas en el suelo, como las tasas de respiración o las actividades enzimáticas (Pérez-De-Mora et al., 2006).

Se investigaron diferentes factores relevantes para la actividad y funcionamiento de la microbiota del suelo. La cobertura vegetal fue uno de los factores clave afectando la actividad biológica de estos suelos. Se encontraron diferencias entre los suelos bajo las tres especies leñosas de estudio (acebuche, álamo blanco y pino piñonero) y con las zonas de pastizal. Los suelos bajo árbol presentaron una mayor relación C:N y una disminución del pH (excepto el pino en la zona Sur), en comparación con los suelos de pastizal. Encontramos un efecto de los árboles en la química del suelo, similar a la encontrada en suelos de bosques leñosos y de pinos (Strickland et al., 2009). Entre las tres especies, el pino piñonero es la que presentó la mayor relación C:N en el suelo, en consonancia con la calidad de la hojarasca que en las coníferas suele estar caracterizada por una relación C:N relativamente alta (Chomel et al., 2015; Cornwell et al., 2008; Pérez-Harguindeguy et al., 2000). Los resultados confirmaron las sólidas evidencias de que los árboles, a través de los exudados de las raíces y la cantidad y calidad de la hojarasca, producen cambios en las propiedades abióticas del suelo (Jones et al., 1994; Mitchell et al., 2010).

Estos cambios químicos conllevan importantes cambios en las comunidades microbianas del suelo. Se observó un efecto positivo de la forestación sobre la biomasa microbiana, que pudo deberse a la mayor biomasa vegetal generada por las especies leñosas, que propicia un aumento de la acumulación de C orgánico y N en el suelo, asociado a un aumento del C y N microbiano, en comparación con los suelos de pastizal.

Las comunidades microbianas también se vieron afectadas por las condiciones del medio. La contaminación, las propiedades abióticas del suelo y la estacionalidad se interrelacionan con el efecto de la cobertura vegetal generando respuestas microbianas muy diversas. Por ejemplo, en sustratos ácidos con bajo contenido en carbonatos el pino

piñonero mostró una mayor capacidad para acidificar el suelo, debido a la baja capacidad de estos suelos para tamponar la acidez. La biomasa microbiana del suelo fue menor en el sitio Norte, posiblemente por el pH ácido y la mayor contaminación.

Las actividades enzimáticas estudiadas se vieron afectadas por la cobertura vegetal, el sitio y la estación del año, mostrando interacciones entre los tres factores, pero con diferentes resultados entre ellas. Las enzimas β-glucosidasa (BGL), fosfatasa ácida (ACP) y N-acetil-glucosaminidasa (NAG) mostraron sus máximas actividades en el sitio Norte en primavera, mientras que la actividad de la leucil-aminopeptidasa (LAP) fue máxima en el sitio Sur en otoño. En cuanto al efecto de la cobertura vegetal, la tendencia general fue de una mayor actividad enzimática en suelos bajo álamo blanco y pino piñonero.

La contaminación por elementos traza puede conllevar una reducción de la actividad total microbiana (Pérez-De-Mora et al., 2006). Sin embargo, los indicadores de actividad total microbiana, diacetato de fluoresceína (FDA) y deshidrogenasa (DH), mostraron resultados opuestos, y no hubo correlación entre ellos o con la biomasa microbiana. Estos indicadores mostraron correlaciones con el pH y los niveles de contaminación del suelo, pero sus tendencias fueron opuestas en relación con el factor sitio y la estacionalidad.

Los cambios estacionales, caracterizados por un aumento de las temperaturas (de aproximadamente 8 ºC) y una reducción de la humedad del suelo (de más del 50%) entre primavera y otoño, indujeron grandes cambios en la actividad de las comunidades microbianas. El C y el N microbiano se mantuvieron con valores similares entre las dos estaciones en el sitio Norte; sin embargo, en el sitio Sur aumentaron mucho en otoño. Este aumento pudo deberse al estímulo microbiano por el aumento de la hojarasca en el otoño, aunque se encontraron diferentes tendencias en los dos sitios de estudio. El cambio estacional de primavera a otoño podría haber propiciado un cambio hacia una mayor dominancia de hongos pudiendo ser este cambio más intenso en el sitio Sur al contar con una mayor humedad y relación C:N (Montiel-Rozas et al., 2018). Mientras que en el sitio Norte los hongos podrían estar más limitados por la acidez o la mayor contaminación de estos suelos, manteniéndose las comunidades microbianas similares a las de primavera. Las actividades enzimáticas mostraron importantes correlaciones con las propiedades abióticas y la contaminación del suelo en primavera, pero en otoño estas correlaciones no fueron significativas, con la excepción de la actividad LAP. Inferimos que en primavera la biomasa y la mayoría de las actividades microbianas están más determinadas y dirigidas por las variables abióticas y la contaminación del suelo; mientras que en otoño aumenta

la estocasticidad. Es posible que en otoño las comunidades microbianas estaban constreñidas por limitaciones fisiológicas o de nutrientes, debido a la reducción de la humedad del suelo, produciendo cambios en las respuestas funcionales o en la composición de la comunidad microbiana (Fierer et al., 2003; Zeglin et al., 2013). Tal vez, ciertas comunidades de hongos, más tolerantes a la disminución de la humedad, se desarrollaron a expensas de otras comunidades bacterianas, más sensibles a la sequía (Aanderud and Lennon, 2011; Aponte et al., 2014; Jensen et al., 2003; Wilkinson et al., 2002). El aumento en la relación C:N de la biomasa microbiana, especialmente en los suelos de la zona Sur, podría ser indicativo de esta tendencia.

La diversidad funcional microbiana, medida a través de la capacidad heterotrófica de las comunidades, no mostró diferencias en el índice de diversidad Shannon. Se observó un incremento de la respiración por degradación de los sustratos acetil glucosamina (AcG), ácido salicílico (SalA) y trehalosa (Tre), bajo las especies leñosas y, especialmente, bajo el álamo blanco. El mayor contenido en humedad y el contenido en N total y C orgánico bajo el álamo blanco parecen explicar tanto el crecimiento de biomasa como la mayor respiración en estos suelos (Bérard et al., 2014). A mayor diversidad en la comunidad microbiana, más amplio el espectro de sustratos respirados (Creamer et al., 2009). Por lo tanto, el aumento en la utilización de los sustratos más recalcitrantes bajo el álamo blanco, podría indicar una comunidad microbiana más diversa funcionalmente bajo esta especie.

En el **Capítulo 5** hemos estudiado los efectos de la fitorremediación en las comunidades fúngicas que se desarrollan en los suelos contaminados del Corredor Verde del Guadiamar. En este estudio hemos observado que el tipo de simbiosis hongo-planta, característica de cada especie vegetal, es determinante en las comunidades de hongos que se desarrollan en cada suelo. Por lo tanto, la cobertura vegetal fue el principal impulsor de la composición y estructura de las comunidades de hongos del suelo, al igual que en anteriores estudios (Buée et al., 2009; Urbanová et al., 2015). Se puede concluir que el efecto específico de cada especie vegetal produce cambios en el suelo, tanto abióticos como bióticos, influyendo a la composición de la comunidad de hongos (Berg y Smalla, 2009; Chaparro et al., 2014; Leff et al., 2018).

La elevada concentración de elementos traza y la escasez de nutrientes encontrada en los suelos que no fueron fitorremediados, inhibieron el desarrollo de las comunidades vegetales y fúngicas. En estos suelos muy contaminados, la vegetación no fue capaz de

establecerse y, además, las comunidades de hongos que allí se desarrollaban tenían, unos 20 años después del episodio de contaminación, una diversidad muy reducida.

En cambio, tras la retirada de los lodos contaminantes y la recuperación del suelo con la aplicación de enmiendas, se promovió el crecimiento vegetal natural estableciéndose comunidades de herbáceas ruderales. Este hábitat de pastizal presentó altos valores de diversidad específica, taxonómica y funcional de hongos, en comparación con los suelos no remediados. La fitorremediación fue la última intervención realizada y consistió en la plantación de diferentes especies de árboles. En nuestro estudio de tres especies leñosas (acebuche, álamo blanco y pino piñonero) descubrimos que el efecto de la especie arbórea en el pH, C y nutrientes del suelo, así como el tipo de interacción simbiótica con los hongos, fueron claves en el desarrollo de las comunidades fúngicas.

Las diferentes coberturas vegetales promueven cambios en las propiedades abióticas del suelo promoviendo diferentes efectos en los hongos (Dickie et al., 2014; Finzi et al., 1998; Wurzburger and Hendrick, 2007). El pH, el contenido en nutrientes y el C estuvieron positivamente correlacionados con la diversidad y la riqueza de especies de hongos. El pH, la textura y la contaminación correlacionaron con la diversidad taxonómica y funcional de las comunidades de hongos. Las principales variables abióticas que determinaron la composición de las comunidades de hongos fueron el C orgánico y la relación C:N del suelo. El acebuche fue la especie leñosa que presentó unas comunidades fúngicas más similares a las de los pastizales, tal vez por sus menores tasas de crecimiento y menor porte en comparación con las otras especies de árboles, pudiendo tener una menor influencia en la micobiota del suelo. En cambio, el pino piñonero presentó una comunidad de hongos muy diferente a la de los pastizales, posiblemente debido a su efecto sobre el suelo: mayor acidificación, alta relación C:N y baja calidad de la hojarasca. El pino piñonero presentó una diversidad y equitabilidad de las comunidades de hongos menor que las de las otras dos especies leñosas, mostrando una dominancia de ciertas especies, explicable por la elevada relación C:N en los suelos bajo coníferas (Ni et al., 2018; Saitta et al., 2018). Este efecto en los hongos del suelo también puede estar propiciado por la acidificación causada por el pino, la cual puede limitar la colonización o el desarrollo de ciertas especies de hongos (Tedersoo et al., 2014; Urbanová et al., 2015).

La riqueza de las comunidades de hongos fue mayor bajo las especies arbóreas que establecen interacciones con hongos ECM: el álamo blanco y el pino piñonero. Además,

estas dos especies presentaron un mayor contenido en C orgánico en el suelo. Los hongos ECM favorecen la supervivencia de los hospedadores mejorando la adquisición de nutrientes y de agua (Tedersoo and Bahram, 2019). En zonas contaminadas, además mitigan la toxicidad de los elementos traza (Huang et al., 2014). Las especies hospedadoras de hongos ECM (pino y álamo) fueron clave en el aumento de la riqueza de las comunidades fúngicas, con cierta dominancia bajo el álamo blanco y el pino piñonero. La mayor acidificación del suelo en el sitio Norte, sin embargo, produjo una reducción de la diversidad de hongos ECM; en general, la diversidad de hongos ECM es menor en suelos ácidos (Tedersoo et al., 2014; Urbanová et al., 2015).

A pesar de la heterogeneidad entre los hábitats de estudio, se observó una pequeña proporción de hongos generalistas comunes a todos los hábitats. Sin embargo, la riqueza y la diversidad global de hongos del suelo fueron similares entre los sitios Norte y Sur, a pesar de las condiciones ambientales contrastadas, con bajo pH y mayor disponibilidad de elementos traza en el sitio Norte. Por lo tanto, el efecto de la cobertura vegetal parece ser más relevante que el de las propiedades abióticas del suelo, en la composición de las comunidades de hongos (Tedersoo et al., 2016).

La composición de la cubierta vegetal también afectó a la riqueza y abundancia relativa de los tipos funcionales de hongos: saprótrofos, patógenos y micorrícicos. Los hongos saprótrofos fueron abundantes en suelos de pastizal y bajo acebuche, mientras que los hongos ECM fueron dominantes bajo los hospedadores álamo blanco y pino piñonero. En los suelos no remediados y con escaso o nulo crecimiento vegetal, los hongos patógenos fueron los más abundantes. Estudios anteriores también mostraron que la composición y riqueza de las especies vegetales influyen en la diversidad y la composición de las comunidades fúngicas del suelo (Gao et al., 2017; Saitta et al., 2018).

En cada hábitat de estudio se descubrieron numerosas especies indicadoras lo que nos habla de la existencia de un filtro ambiental que favorece un mayor desarrollo de ciertas especies de hongos mediante su adaptación a cada hábitat (Dufrêne and Legendre, 1997). En el caso de los suelos no remediados y muy contaminados, se descubrió la presencia de especies que son indicadoras de condiciones ambientales extremas. Probablemente, gracias a la fitorremediación de los suelos contaminados, estas comunidades extremófilas pudieron ser reemplazadas por otras comunidades de hongos más competitivos y más generalistas, que tomaron ventaja de las condiciones más favorables del suelo,

favoreciendo el desarrollo de la vegetación y la remediación del ecosistema (Hujslová and Gryndler, 2019).

En el **Capítulo 6** hemos estudiado los efectos de la contaminación por elementos traza en la composición de las comunidades de hongos ECM en simbiosis con la encina, así como en sus rasgos morfológicos. Los rasgos medidos en los hongos están relacionados con el tipo exploratorio y el contenido en melanina; mostraron ser una buena herramienta para cuantificar los efectos potenciales de la perturbación ambiental en las comunidades fúngicas del suelo.

La contaminación no pareció afectar a la riqueza específica de hongos ECM asociados a cada árbol. La baja riqueza relativa de especies ECM y el bajo efecto de las variables abióticas en la composición de las comunidades ECM podrían ser explicadas por la juventud de las plantaciones. Podríamos estar en un escenario de sucesión primaria donde los procesos estocásticos son los que dirigen el ensamblado de las comunidades (Jumpponen, 2003; Kennedy et al., 2009; Peay y Bruns, 2014).

A pesar de que la influencia de las variables abióticas en las riqueza específica de ECM fue baja, estas variables, distinguiendo entre concentración de elementos traza y propiedades generales del suelo, explicaron la misma proporción en la composición de especies. El gradiente de contaminación y el gradiente de contenido en Ca del suelo mostraron relación con la presencia de las dos especies ECM más abundantes, *Hebeloma cavipes* y *Thelephora terrestris*, respectivamente.

La contaminación pareció afectar más a los rasgos morfológicos medios de las comunidades de hongos y a la similaridad de estos rasgos que a la diversidad taxonómica de la especies de ECM. La contaminación parece tener un efecto supresor en la frecuencia de rizomorfos y de hifas emanentes. Las hifas mostraron una relación negativa con el C total del suelo, y los rizomorfos con la disponibilidad de nitrato, lo cual podría explicarse por la reducción del desarrollo del micelio cuando hay un cambio de fuentes de N inorgánicas a orgánicas (Hobbie and Agerer, 2010; Lilleskov et al., 2011, 2002). El grado de melanización no mostró una relación con la contaminación, como se esperaba (Gadd and de Rome, 1988); sin embargo, mostró una relación positiva con el contenido en $CaCO_3$, que parece indicar una implicación de la melanina en la regulación del Ca en las células del hongo (Bush et al., 2007).

La variación de las medias ponderadas para la comunidad (CWM) de los rasgos fúngicos medidos fue explicada dos veces más por la contaminación que por el resto de propiedades abióticas del suelo. La contaminación determinó la dispersión de los rasgos morfológicos de los hongos. Se propone la existencia de un filtro ambiental de la contaminación sobre estos rasgos; al aumentar la concentración de Cd en el suelo el grado de diversidad de los rizomorfos disminuyó. Esto nos indica que la contaminación puede inducir una convergencia en los rasgos morfológicos y, en consecuencia, disminuye la diversidad funcional de los hongos ECM (Bässler et al., 2015). En cuanto a las hifas emanentes, mostraron (de manera marginal) una divergencia con el aumento de la concentración de Cd en el suelo. Esto podría explicarse por una interacción entre ambos rasgos; primero, la comunidad ECM es filtrada en cuanto a su producción de rizomorfos y, después, las especies se seleccionan por competición (Ingram y Shurin, 2009). Las consecuencias de la reducción de la diversidad funcional de las comunidades de hongos ECM pueden producir efectos en el funcionamiento de la planta y del ecosistema. Por ejemplo, se conoce que el contenido en melanina y la red de hifas afectan a la descomposición y al almacenamiento de C en el suelo (Clemmensen et al., 2015; Fernandez and Kennedy, 2016), además de su importante papel en la adquisición de agua (Fernandez and Koide, 2014), tan crucial en ambientes mediterráneos. Este estudio confirma el interés de utilizar un enfoque basado en los rasgos morfológicos de los hongos ECM para conocer las consecuencias de la contaminación por elementos traza en estas comunidades.

En el **Capítulo 7** hemos estudiado la influencia de las comunidades de hongos ECM en algunos rasgos morfológicos y químicos de la encina. Como hemos visto en el Capítulo 6, las propiedades abióticas del suelo modulan las comunidades de hongos ECM, especialmente los rasgos morfológicos de estos organismos. Debido a esta influencia del suelo en las comunidades de hongos ECM, los efectos producidos por el suelo en los rasgos de la encina estarán también mediados por los hongos ECM en la relación suelo-planta. En este estudio, encontramos que tanto la composición de hongos ECM como sus rasgos funcionales fueron más explicativos de los rasgos de la encina que las propiedades del suelo.

Los sistemas radiculares muestran una alta plasticidad según la heterogeneidad del suelo (Ostonen et al., 2007). En el gradiente ambiental estudiado, los rasgos morfológicos de

las raíces fueron altamente explicados por la composición de especies y los rasgos de los hongos ECM; estos resultados corroboran la importancia de incorporar los rasgos micorrícicos en los análisis de rasgos funcionales en raíces (Weemstra et al., 2016). En cuanto al "espectro económico de las raíces", encontramos que las raíces de las encinas donde colonizaban los hongos ECM más abundantes (*H. cavipes* y *T. terrestris*) mostraban alta relación C:N, bajo contenido en N y P, alto contenido en materia seca (RDMC) y baja área específica (SRA), que corresponden a una estrategia "conservativa" de recursos (de la Riva et al., 2018, 2016; Marañón et al., 2020). Los rasgos de estas especies abundantes de hongos (*H. cavipes* y *T. terrestris*) se caracterizaron por una alta formación de rizomorfos y un bajo contenido en melanina. En el otro lado del "espectro económico de las raíces", las raíces que fueron colonizadas por especies de hongos ECM más raras mostraron alto contenido en N y P, bajo RDMC y alto SRA, que corresponden a una estrategia "adquisitiva" de recursos (de la Riva et al., 2018, 2016; Marañón et al., 2020). Esto puede indicar que cuando las condiciones son más favorables, con una menor limitación de recursos, estas encinas pueden tener una menor dependencia de la formación de rizomorfos en los hongos ECM de su rizosfera. Por otra parte, en condiciones menos favorables, con una mayor concentración de contaminantes, puede existir la necesidad de evitar su toxicidad aumentando la producción de melanina en estos hongos.

Cabría esperar que en las zonas más contaminadas las raíces mostraran rasgos más conservativos, pero sin embargo no encontramos esta tendencia. Podría ser que el principal factor de estrés edáfico para la encina en la zona de estudio no es la contaminación, sino una limitación en nutrientes o agua. Tal vez la contaminación del suelo es independiente del "espectro económico de las raíces", indicando que existe un marco multidimensional que incluye otros procesos aparte de los relacionados con la nutrición (Weemstra et al., 2016). A pesar de los niveles altos de contaminación del suelo en el extremo del gradiente ambiental estudiado , la transferencia de elementos traza a las hojas fue baja (excepto para el Mn), por lo que la encina se puede considerar una especie con un potencial fitoestabilizador de elementos traza (Domínguez et al., 2009). La transferencia de As, Mn y Zn del suelo a las raíces fue altamente explicada por las especies ECM y sus rasgos, por lo que los hongos ECM parecen mediar en la retención de los elementos traza en esta especie arbórea. Sin embargo, no todos los elementos traza mostraron las mismas relaciones, por lo que los mecanismos de transferencia y retención deben ser específicos (Godbold et al., 1998; Jentschke and Godbold, 2000). Las raíces están constreñidas por diversos factores abióticos y bióticos (Weemstra et al., 2016); por

otra parte, en un suelo contaminado por multitud de elementos traza estos pueden afectar de diversas maneras a los rasgos de los hongos ECM asociados a las raíces.

En la encina se descubrió una relación positiva entre la composición de especies ECM asociadas al árbol y el C, el P y las relaciones C:N y N:P de las hojas. Por lo tanto, puede existir un efecto indirecto de la comunidad de hongos ECM en la nutrición de la planta a través de los efectos de estos hongos en los rasgos radiculares. También encontramos una relación positiva del P del suelo con los valores de N, P y clorofila (CCI) en las hojas, pudiendo indicar una limitación de P en estos suelos, ya detectada en estudios anteriores realizados en la zona (Domínguez et al., 2010). Sin embargo, algunos rasgos morfológicos de las hojas, como el área foliar específica (SLA) y el contenido de clorofila, no mostraron relaciones con los hongos ECM; posiblemente porque son más dependientes del proceso de fotosíntesis (Niinemets and Sack, 2006).

Finalmente, en el **Capítulo 8** hemos estudiado las diferencias en la acumulación de elementos traza y los mecanismos de nutrición entre dos especies de setas silvestres recolectadas en el Corredor Verde del Guadiamar.

El micelio de los hongos tiene una alta capacidad de retención de diferentes elementos traza, lo cual es beneficioso para estabilizarlos en el suelo (micoestabilización). Sin embargo, estos elementos pueden transferirse y acumularse en los cuerpos fructíferos pudiendo implicar un riesgo potencial por toxicidad si son consumidos (Purohit et al., 2018). En este estudio comparamos los mecanismos de nutrición del hongo saprofítico (*Volvopluteus gloiocephalus*) y del hongo ECM (*Laccaria laccata*) mediante el análisis de sus carpóforos (setas). Aunque ambas especies de setas se recolectaron en la misma zona de estudio, presentaron diferencias en las condiciones ambientales en las que fueron encontradas. Los suelos alrededor de las setas de *V. gloiocephalus* presentaron mayores concentraciones *pseudo-totales* de Cd, Co, Mn y Zn, mientras que las concentraciones *disponibles* de elementos traza fueron mayores en suelos bajo las setas de *L. laccata.* Estas diferencias pueden deberse a que las setas de *L. laccata* estaban asociadas al pino, el cual tiene un efecto acidificante en el suelo (**Capítulo 4**) aumentando la disponibilidad de los elementos traza (Madejón et al., 2018), mientras que las setas de *V. gloiocephalus* crecieron en zonas de pastizal. Tanto la diferencia en el hábito como la variedad en el hábitat parecen influir sobre la nutrición de los hongos. Las setas de *L. laccata* (micorrícica) presentaron un menor contenido nutricional que las setas de *V.*

gloiocephalus (saprofítica), probablemente debido a la transferencia de parte del N y P asimilado por el hongo a la planta, a cambio de C de la planta al hongo. Esta diferencia nutricional se investigó con el estudio de isótopos de C y N. Las setas de *L. laccata* mostraron valores más reducidos en ^{13}C que en su suelo correspondiente, ya que reciben el C de la fotosíntesis de la planta hospedadora; mientras que estaban enriquecidas en ^{15}N por la discriminación isotópica de los procesos biológicos (Hobbie et al., 1999; Rosling et al., 2004). En cambio, las setas de *V. gloiocephalus* mostraron valores de ^{13}C similares a los del suelo (su fuente de C); mientras que los valores de ^{15}N fueron menores, por la preferencia de uso de ^{14}N en los procesos biológicos (Dawson et al., 2002).

La capacidad de los hongos para acumular elementos traza en su micelio y en sus cuerpos fructíferos puede explicar las relaciones hongo-suelo con los diferentes elementos traza. Encontramos que la retención de elementos traza depende tanto del contaminante como de la especie de hongo. El conocimiento sobre los mecanismos del transporte de metales del micelio a la seta aún son limitados (Kalač and Svoboda, 2000). Sin embargo, los elementos traza encontrados en la seta provienen del micelio, por lo tanto, parte de estos elementos deben estar movilizados en el micelio, el cual podría fitoestabilizar en el suelo elementos potencialmente tóxicos. Sin embargo, la emergencia de los cuerpos fructíferos podría suponer un riesgo toxicológico si fueran ingeridos. En este estudio, ambas especies de setas bioacumularon principalmente Cd; a pesar de una mayor disponibilidad de Cd en suelos cercanos a *L. laccata*, las setas de *V. gloiocephalus* acumularon significativamente más Cd, pudiendo deberse a la competencia que se genera entre los elementos Cd-Zn-Ca por el uso de los mismos transportadores (Hartley et al., 1997). Las propiedades abióticas del suelo como el pH, la textura y el contenido en materia orgánica, entre otros, producen cambios en la solución del suelo afectando a la absorción de metales en las setas (Elekes et al., 2010). En nuestro estudio, se observó que la bioacumulación de elementos traza en las setas estaba influenciada por algunas propiedades abióticas del suelo, principalmente pH, Ca y K.

Aparte de la acumulación de Cd, los valores de Cu, Mn, Zn y Fe en las setas también fueron elevados. Por ello, analizamos si existía algún riesgo de toxicidad por el consumo de estas setas. En cuanto a las concentraciones límites en setas reguladas por la Unión Europea, sólo existen límites para los elementos Pb y Cd; encontramos que para ninguno de estos elementos se alcanzaban los niveles máximos permitidos en las setas analizadas (European Commission, 2015, 2014). También se analizaron los límites de ingesta tolerables establecidos por la Organización Mundial de la Salud (WHO, 2017);

encontramos que con una ingesta diaria de 0.069 kg en peso fresco de *V. gloiocephalus* o de 0.132 kg en peso fresco de *L. laccata* se alcanzaría la ingesta máxima tolerable diaria de Cd, sin incluir otras fuentes de Cd. Comparado con las concentraciones encontradas en setas en otros estudios (Kalač, 2010; Kalač and Svoboda, 2000), el consumo de estas setas, especialmente de *V. gloiocephalus* podría representar un riesgo toxicológico si fueran consumidas.

9.2 Síntesis de los resultados de la Tesis Doctoral

En esta tesis se aplican diversos enfoques y técnicas de investigación para abordar las interacciones entre suelo, hongos y plantas en una zona contaminada y fitorremediada. Se aportan datos novedosos que contribuyen al conocimiento del sistema complejo y que pueden facilitar a los gestores la toma de decisiones sobre la conservación de este espacio protegido y de futuros espacios degradados.

Los estudios realizados en esta Tesis Doctoral nos han permitido conocer cómo evoluciona el proceso de restauración en este ecosistema terrestre a través del estudio de las comunidades microbianas y los procesos ecosistémicos en los que participan.

Hemos descubierto la importancia de la selección de las especies utilizadas en la forestación en suelos contaminados por elementos traza. En las interacciones planta-suelo se produce una retroalimentación que conlleva unos cambios en las propiedades abióticas del suelo por parte de la vegetación y estos cambios producen un efecto en las comunidades microbianas, las cuales influyen, a su vez, en el estado de la vegetación. A pesar de los intrincados mecanismos y relaciones, en esta Tesis Doctoral, hemos podido esclarecer más estas relaciones.

Las medidas de fitorremediación aplicadas en el Corredor Verde del Guadiamar parecen haber sido esenciales para el establecimiento de la vegetación y de sus comunidades microbianas asociadas. Las comunidades microbianas, incluidos los hongos, parecen estar más afectados por la comunidad de plantas que por las características abióticas del suelo. La forestación de especies leñosas implicó un mayor porcentaje de C orgánico, N y relación C:N en los suelos, especialmente bajo álamo y pino.

Entre las especies estudiadas, el acebuche presentó el menor efecto en el suelo comparado con el álamo y el pino. En estos suelos se produjo una mayor acumulación de N y un

aumento del C de la biomasa microbiana. Sin embargo, la actividad enzimática y la diversidad catabólica no fueron muy elevadas en estos suelos. En cuanto a las comunidades de hongos, se encontró una alta diversidad pero una baja riqueza de especies y de familias. Los hongos más abundantes fueron los saprótrofos, al igual que en suelos de pastizal. En general, los suelos bajo acebuche y de pastizal presentaron características y comunidades microbianas (y fúngicas) similares.

El pino produjo un aumento en el contenido en C orgánico y N y, especialmente, en la relación C:N. En cuanto a la actividad microbiana, esta especie presentó una alta biomasa de C y una elevada actividad enzimática (fosfatasa ácida, leucil aminopeptidasa y N-acetil glucosaminidasa). Sin embargo, el suelo bajo pino presentó una reducida actividad deshidrogenasa.

El álamo blanco fue una especie con importantes efectos en el suelo mediante el aumento del C orgánico, el N y la relación C:N. Además, el álamo fue capaz de mantener una mayor humedad en el suelo favoreciendo, junto con otras propiedades, una mayor diversidad catabólica y, por lo tanto, funcional en estos suelos. Se encontraron elevados valores de biomasa microbiana, tanto de C como de N, y de las actividades enzimáticas (beta glucosidasa, leucil aminopeptidasa y N- acetil glucosaminidasa). En cuanto a la comunidad de hongos, fueron los suelos más favorables para la diversidad y para la riqueza de especies y de familias.

El tipo de simbiosis que se establece entra la vegetación y los hongos micorrícicos parece clave en la composición de las comunidades fúngicas. Tanto el álamo como el pino presentaron una comunidad de hongos más similar que la del acebuche, debido a que comparten la misma simbiosis con hongos ectomicorrícicos, presentando un elevado número de especies ectomicorrícicas en estos suelos. Entre estas dos especies, el pino presentó una menor equitabilidad, indicando que existe cierta dominancia de algunas especies.

Debido a los cambios positivos que producen tanto el álamo como el pino en los suelos, se podría pensar en que estas especies son adecuadas para su forestación en futuras estrategias de fitorremediación de suelos contaminados por elementos traza. En términos generales, podríamos indicar que el álamo es la especie que mejor promueve la restauración del ecosistema mediante un aumento de la materia orgánica y de la actividad microbiana. Sin embargo, se conoce que esta especie tiene la capacidad de movilizar y acumular Cd y Zn en sus hojas y hojarasca, por lo que se favorecería la movilidad de estos

elementos. El pino también podría considerarse una especie adecuada para la restauración debido a sus cambios en las propiedades tanto abióticas como bióticas. Sin embargo, en suelos ácidos y con bajo contenido en carbonatos, el pino produce una mayor acidificación en el suelo lo que fomentaría la disponibilidad y movilidad de los elementos traza. El pino sí sería una especie adecuada en la forestación de suelos con un alto contenido en carbonatos donde el efecto acidificador de esta especie pudiera tamponarse y así evitar una mayor disponibilidad de elementos traza. En cuanto al acebuche, esta especie no produjo un gran efecto en los suelos, ni químicamente ni biológicamente, pero tampoco se conocen efectos adversos de esta especie en la movilización de elementos traza.

El estudio de los hongos ectomicorrícicos en simbiosis con la encina, confirma el importante papel de estos organismos en la restauración y en el establecimiento de la vegetación, mediante mecanismos de mediación y protección a los árboles.

Aparte del papel de las especies que forman estas comunidades, el estudio de algunos de sus rasgos morfológicos nos hablan de la importancia de incluir los rasgos en los estudios fúngicos. La reducción en la translocación de los elementos traza y la mejora de los estados nutricionales de la planta, son algunos de los mecanismos en los que estos rasgos pueden estar implicados. Tanto la contaminación como las propiedades del suelo determinaron en cierta medida la composición de hongos del suelo, mostrando relación con la presencia de las dos especies ECM más abundantes, *Hebeloma cavipes* y *Thelephora terrestris*. Sin embargo, la contaminación mostró un mayor efecto en los rasgos morfológicos de los hongos relacionados con el tipo exploratorio (hifas emanentes y rizomorfos) y el contenido en melanina, que en la diversidad taxonómica. La contaminación podría tener un efecto supresor en la frecuencia de rizomorfos y de hifas emanentes, además de producir comunidades con rasgos más convergentes y, por lo tanto, menos funcionales. Una reducción de la diversidad funcional de las comunidades de hongos ECM puede producir efectos en el funcionamiento de la planta y del ecosistema.

Debido al efecto del suelo en los hongos ectomicorrícicos y debido a la simbiosis existente entre la encina y estos hongos, el efecto del suelo en la encina podría estar mediado por los hongos ectomicorrícicos. Se estudiaron algunos rasgos morfológicos y químicos de las raíces y hojas de la encina; tanto la comunidad como los rasgos morfológicos de los hongos ectomicorrícicos determinaban el estado de la encina en mayor medida que las características del suelo. Los hongos ectomicorrícicos son

interesantes de estudiar en los análisis de rasgos funcionales en raíces ya que encontramos que están relacionados con el "espectro económico de las raíces". Por un lado, las raíces de las encinas donde colonizaban los hongos ectomicorrícicos más abundantes (*H. cavipes* y *T. terrestris*) caracterizados por la formación de rizomorfos y un bajo contenido en melanina se correspondieron a una estrategia "conservativa" en el uso de los recursos. Por otro lado, las raíces que fueron colonizadas por especies de hongos ectomicorrícicos más raras, con una menor formación de rizomorfos, se correspondieron a una estrategia "adquisitiva" en el uso de los recursos. Los hongos ectomicorrícicos parecen mediar en la retención de los elementos traza en las raíces de la encina, siendo la transferencia a las hojas baja (excepto el Mn). Por lo tanto, la encina se podría considerar una especie adecuada para la forestación de suelos contaminados por elementos traza debido a su potencial fitoestabilizador.

Esta alta capacidad de retener elementos traza junto con la concentración de la mayor parte de su biomasa en el micelio, confiere a los hongos una alta capacidad de estabilizar la contaminación, lo que se conoce como micoestabilización. Sin embargo, los cuerpos fructíferos epígeos (setas) pueden acumular estos elementos y ser transferidos a la cadena trófica a través de su consumo. Las setas de *Laccaria laccata* presentaron una mayor concentración de elementos traza que las de *Volvopluteus gloiocephalus* debido a su simbiosis con el pino, establecido en suelos ácidos donde la disponibilidad de estos elementos era mayor. La bioacumulación de elementos traza en las setas estaba influenciada principalmente por el pH, Ca y K. Se encontró que ambas especies de setas bioacumulaban Cd con un riesgo potencial de toxicidad por su consumo.

9.3 Perspectivas futuras de investigación derivadas de esta Tesis Doctoral

La presente Tesis Doctoral ha contribuido de manera novedosa a un mayor conocimiento del papel de las comunidades microbianas y, especialmente, de las comunidades de hongos en la restauración de zonas degradadas y contaminadas.

Esta Tesis Doctoral ha contribuido a un mayor conocimiento de los efectos que producen diferentes coberturas vegetales en el suelo, tanto química como biológicamente. En el futuro, se podrían establecer estudios más exhaustivos: aumentando las especies de

estudio o seleccionando las especies más representativas de cada ecosistema, para tener más información sobre diferentes especies que pudieran ayudar a futuros planes de fitorremediación en otras zonas degradadas o contaminadas.

Debido a que esta Tesis Doctoral tiene un límite tanto espacial como temporal, sería interesante realizar estudios más globales comparando con otras zonas que también se han visto afectadas por la contaminación para contrastar con nuestros resultados. Y a la vez, mantener una evaluación en el tiempo del Corredor Verde del Guadiamar para conocer la evolución de las comunidades microbianas en el tiempo, junto con la monitorización de los niveles de contaminación por elementos traza.

En la actualidad, a pesar de la rápida evolución de las tecnologías "ómicas", las comunidades microbianas del suelo son aún bastante desconocidas. En esta Tesis Doctoral hemos descubierto el importante papel de los hongos en las interacciones planta-suelo pero aún son necesarios más estudios que clarifiquen la importancia de la microbiota en general y de los hongos en particular. La evaluación no sólo de las comunidades sino de sus rasgos morfológicos parece ser de gran importancia en estas interacciones, por lo que estudios enfocados a determinar los rasgos fúngicos, son de elevado interés.

Por último, es importante que se produzca una monitorización de estas zonas contaminadas con estudios a largo plazo, debido a su escasez, y debido a los efectos adversos que se pueden seguir produciendo en estos ecosistemas tiempo después del evento catastrófico, como hemos mostrado a través del estudio de los cuerpos fructíferos.

9.4 Bibliografía

Aanderud, Z.T., Lennon, J.T., 2011. Validation of heavy-water stable isotope probing for the characterization of rapidly responding soil bacteria. Applied and Environmental Microbiology 77, 4589-4596.

Aponte, C., Matías, L., González-Rodríguez, V., Castro, J., García, L. V., Villar, R., Marañón, T., 2014. Soil nutrients and microbial biomass in three contrasting Mediterranean forests. Plant and Soil 380, 57-72.

Bardgett, R.D., van der Putten, W.H., 2014. Belowground biodiversity and ecosystem functioning. Nature 515, 505-511.

Bässler, C., Heilmann-Clausen, J., Karasch, P., Brandl, R., Halbwachs, H., 2015. Ectomycorrhizal fungi have larger fruit bodies than saprotrophic fungi. Fungal Ecology 17, 205-212.

Berg, G., Smalla, K., 2009. Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. FEMS Microbiology Ecology 68, 1-13.

Buée, M., Reich, M., Murat, C., Morin, E., Nilsson, R.H., Uroz, S., Martin, F., 2009. 454 Pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. New Phytologist 184, 449-456.

Burns, R.G., DeForest, J.L., Marxsen, J., Sinsabaugh, R.L., Stromberger, M.E., Wallenstein, M.D., Weintraub, M.N., Zoppini, A., 2013. Soil enzymes in a changing environment: Current knowledge and future directions. Soil Biology and Biochemistry 58, 216-234.

Bush, W.D., Simon, J.D. 2007. Quantification of Ca^{2+} binding to melanin supports the hypothesis that melanosomes serve as functional role in regulating calcium homeostasis. Pigment Cell Research, 20, 134-139

Cabral, L., Soares, C.R.F.S., Giachini, A.J., Siqueira, J.O., 2015. Arbuscular mycorrhizal fungi in phytoremediation of contaminated areas by trace elements: mechanisms and major benefits of their applications. World Journal of Microbiology and Biotechnology 31, 1655-1664.

Chaparro, J.M., Badri, D. V., Vivanco, J.M., 2014. Rhizosphere microbiome assemblage is affected by plant development. ISME Journal 8, 790-803.

Chomel, M., Guittonny-Larchevêque, M., DesRochers, A., Baldy, V., 2015. Home field advantage of litter decomposition in pure and mixed plantations under boreal climate. Ecosystems 18, 1014-1028.

Clemmensen, K.E., Finlay, R.D., Dahlberg, A., Stenlid, J., Wardle, D.A., Lindahl, B.D., 2015. Carbon sequestration is related to mycorrhizal fungal community shifts during long-term succession in boreal forests. New Phytologist 205, 1525-1536.

Cornwell, W.K., Cornelissen, J.H.C., Amatangelo, K., Dorrepaal, E., Eviner, V.T., Godoy, O., Hobbie, S.E., Hoorens, B., Kurokawa, H., Pérez-Harguindeguy, N., Quested, H.M., Santiago, L.S., Wardle, D.A., Wright, I.J., Aerts, R., Allison, S.D., Van Bodegom, P., Brovkin, V., Chatain, A., Callaghan, T. V., Díaz, S., Garnier, E., Gurvich, D.E., Kazakou, E., Klein, J.A., Read, J., Reich, P.B., Soudzilovskaia, N.A., Vaieretti, M.V., Westoby, M., 2008. Plant species traits are the predominant control on litter decomposition rates within biomes worldwide. Ecology Letters 11, 1065-1071.

Creamer, R.E., Stone, D., Berry, P., Kuiper, I., 2016. Measuring respiration profiles of soil microbial communities across Europe using MicroRespTM method. Applied Soil Ecology 97, 36-43.

Dawson, T.E., Mambelli, S., Plamboeck, A.H., Templer, P.H., Tu, K.P., 2002. Stable isotopes in plant ecology. Annual Review of Ecology and Systematics 33, 507-559.

de la Riva, E.G., Marañón, T., Pérez-Ramos, I.M., Navarro-Fernández, C.M., Olmo, M., Villar, R., 2018. Root traits across environmental gradients in Mediterranean woody communities: are they aligned along the root economics spectrum? Plant and Soil 1-14.

de la Riva, E.G., Tosto, A., Pérez-Ramos, I.M., Navarro-Fernández, C.M., Olmo, M., Anten, N.P.R., Marañón, T., Villar, R., 2016. A plant economics spectrum in Mediterranean forests along environmental gradients: Is there coordination among leaf, stem and root traits? Journal of Vegetation Science 27, 187-199.

Dick, W.A.D., Tabatabai, M.A., 1992. Significance and potential uses of soil enzymes. Soil microbial ecology 95-127.

Dickie, I.A., Koele, N., Blum, J.D., Gleason, J.D., McGlone, M.S., 2014. Mycorrhizas in changing ecosystems. Botany 92, 149-160.

Domínguez, M.T., Madrid, F., Marañón, T., Murillo, J.M., 2009. Cadmium availability in soil and retention in oak roots: potential for phytostabilization. Chemosphere 76, 480-486.

Domínguez, M.T., Marañón, T., Murillo, J.M., Schulin, R., Robinson, B.H., 2010. Nutritional status of Mediterranean trees growing in a contaminated and remediated area. Water, Air, and Soil Pollution 205, 305-321.

Dufrêne, M., Legendre, P., 1997. Species assemblages and indicator species: the need for a flexible asymmetrical approach. Ecological Monographs 67, 345-366.

Elekes, C.C., Busuioc, G., Ionita, G., 2010. The bioaccumulation of some heavy metals in the fruiting body of wild growing mushrooms. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 38, 147-151.

European Commission, 2015. Commission Regulation (EU) 2015/1005 of 25 June 2015 amending Regulation (EC) No 1881/2006 as regards maximum levels of lead in certain foodstuffs, Official Journal of the European Union.

European Commission, 2014. Commission Regulation (EU) No 488/2014 of 12 May 2014 amending Regulation (EC) No 1881/2006 as regards maximum levels of cadmium in foodstuffs, Official Journal of the European Union.

Fernandez, C.W., Kennedy, P.G., 2016. Revisiting the «Gadgil effect»: Do interguild fungal interactions control carbon cycling in forest soils? New Phytologist 209, 1382-1394.

Fernandez, C.W., Koide, R.T., 2014. Initial melanin and nitrogen concentrations control the decomposition of ectomycorrhizal fungal litter. Soil Biology and Biochemistry 77, 150-157.

Fierer, N., Schimel, J.P., Holden, P.A., 2003. Influence of drying-rewetting frequency on soil bacterial community structure. Microbial Ecology 45, 63-71.

Finzi, A.C., Van Breemen, N., Canham, C.D., 1998. Canopy tree-soil interactions within temperate forests: species effects on pH and cations. Ecological Applicaitons 8, 440-446.

Gao, C., Shi, N.N., Chen, L., Ji, N.N., Wu, B.W., Wang, Y.L., Xu, Y., Zheng, Y., Mi, X.C., Ma, K.P., Guo, L.D., 2017. Relationships between soil fungal and woody plant assemblages differ between ridge and valley habitats in a subtropical mountain forest. New Phytologist 213, 1874-1885.

Godbold, D.L., Jentschke, G., Winter, S., Marschner, P., 1998. Ectomycorrhizas and amelioration of metal stress in forest trees. Chemosphere 36, 757-762.

Hartley, J., Cairney, J.W.G., Sanders, F.E., Meharg, A.A., 1997. Toxic interactions of metal ions (Cd2+, Pb2+, Zn2+ and Sb3-) on in vitro biomass production of ectomycorrhizal fungi. New Phytologist 137, 551-562.

Hobbie, E.A., Agerer, R., 2010. Nitrogen isotopes in ectomycorrhizal sporocarps correspond to belowground exploration types. Plant and Soil 327, 71-83.

Hobbie, E.A., Macko, S.A., Shugart, H.H., 1999. Insights into nitrogen and carbon dynamics of ectomycorrhizal and saprotrophic fungi from isotopic evidence. Oecologia 118, 353.

Huang, J., Nara, K., Zong, K., Wang, J., Xue, S., Peng, K., Shen, Z., Lian, C., 2014. Ectomycorrhizal fungal communities associated with Masson pine (Pinus massoniana) and white oak (Quercus fabri) in a manganese mining region in Hunan Province, China. Fungal Ecology 9, 1-10.

Hujslová, M., Gryndler, M., 2019. Fungi in biofilms of highly acidic soils, en: Tiquia-Arashiro, S.M., Grube, M. (Eds.), Fungi in extreme environments: ecological role and biotechnological significance. Springer Nature Switzerland, Cham, pp. 185-203.

Ingram, T., Shurin, J.B. 2009. Trait-based assembly and phylogenetic structure in northeast Pacific rockfish assemblages. Ecology, 90, 2444–2453.

Jumpponen, A. 2003. Soil fungal community assembly in a primary successional glacier forefront ecosystem as inferred from rDNA sequence analyses. New Phytologist, 158, 569–578.

Jensen, K.D., Beier, C., Michelsen, A., Emmett, B.A., 2003. Effects of experimental drought on microbial processes in two temperate heathlands at contrasting water conditions. Applied Soil Ecology 24, 165-176.

Jentschke, G., Godbold, D.L., 2000. Metal toxicity and ectomycorrhizas. Physiologia Plantarum 109, 107-116.

Jones, C.G., Lawton, J.H., Shachak, M., 1994. Organisms as ecosystem engineers. Oikos 69, 373-386.

Jumpponen, A., 2003. Soil fungal community assembly in a primary successional glacier forefront ecosystem as inferred from rDNA sequence analyses. New Phytologist 158, 569-578.

Kalač, P., 2010. Trace element contents in European species of wild growing edible mushrooms: A review for the period 2000-2009. Food Chemistry 122, 2-15.

Kalač, P., Svoboda, L., 2000. A review of trace element concentrations in edible mushrooms. Food Chemistry 69, 273-281.

Kennedy, P.G., Peay, K.G., Bruns, T.D., 2009. Root tip competition among ectomycorrhizal fungi: are priority effects a rule or an exception? Ecology 90, 2098-2107.

Khan, A.G., 2005. Role of soil microbes in the rhizospheres of plants growing on trace metal contaminated soils in phytoremediation. Journal of Trace Elements in Medicine and Biology 18, 355-364.

Krumins, J.A., Goodey, N.M., Gallagher, F., 2015. Plant-soil interactions in metal contaminated soils. Soil Biology and Biochemistry 80, 224-231.

Leff, J.W., Bardgett, R.D., Wilkinson, A., Jackson, B.G., Pritchard, W.J., De Long, J.R., Oakley, S., Mason, K.E., Ostle, N.J., Johnson, D., Baggs, E.M., Fierer, N., 2018. Predicting the structure of soil communities from plant community taxonomy, phylogeny, and traits. ISME Journal 12, 1794-1805.

Lilleskov, E.A., Hobbie, E.A., Fahey, T.J., 2002. Ectomycorrhizal Fungal Taxa Differing in Response to Nitrogen Deposition also Differ in Pure Culture Organic Nitrogen Use and Natural Abundance of Nitrogen Isotopes. New Phytologist 154, 219-231.

Lilleskov, E.A., Hobbie, E.A., Horton, T.R., 2011. Conservation of ectomycorrhizal fungi: Exploring the linkages between functional and taxonomic responses to anthropogenic N deposition. Fungal Ecology 4, 174-183.

Madejón, P., Domínguez, M.T., Gil-Martínez, M., Navarro-Fernández, C.M., Montiel-Rozas, M.M., Madejón, E., Murillo, J.M., Cabrera, F., Marañón, T., 2018. Evaluation of amendment addition and tree planting as measures to remediate contaminated soils: the Guadiamar case study (SW Spain). Catena 166, 34-43.

Marañón, T., Navarro-Fernández, C. M., Gil-Martínez, M., Domínguez, M. T., Madejón, P., Villar, R., 2020. Variation in morphological and chemical traits of Mediterranean tree roots: linkage with leaf traits and soil conditions. Plant and Soil, 449, 389–403.

Mitchell, R.J., Campbell, C.D., Chapman, S.J., Cameron, C.M., 2010. The ecological engineering impact of a single tree species on the soil microbial community. Journal of Ecology 98, 50-61.

Ni, Y., Yang, T., Zhang, K., Shen, C., Chu, H., 2018. Fungal communities along a small-scale elevational gradient in an alpine tundra are determined by soil carbon nitrogen ratios. Frontiers in Microbiology 9, 1815.

Niinemets, Ü., Sack, L., 2006. Structural determinants of leaf light-harvesting capacity and photosynthetic potentials. Progress in Botany 67, 385-419.

Op De Beeck, M., Lievens, B., Busschaert, P., Rineau, F., Smits, M., Vangronsveld, J., Colpaert, J. V., 2015. Impact of metal pollution on fungal diversity and community structures. Environmental Microbiology 17, 2035-2047.

Ostonen, I., Püttsepp, Ü., Biel, C., Alberton, O., Bakker, M.R., Lõhmus, K., Majdi, H., Metcalfe, D., Olsthoorn, A.F.M., Pronk, A., Vanguelova, E., Weih, M., Brunner, I., 2007. Specific root length as an indicator of environmental change. Plant Biosystems 141, 426-442.

Peay, K.G., Bruns, T.D., 2014. Spore dispersal of basidiomycete fungi at the landscape scale is driven by stochastic and deterministic processes and generates variability in plant-fungal interactions. New Phytologist 204, 180-191.

Pérez-De-Mora, A., Burgos, P., Madejón, E., Cabrera, F., Jaeckel, P., Schloter, M., 2006. Microbial community structure and function in a soil contaminated by heavy metals: Effects of plant growth and different amendments. Soil Biology and Biochemistry 38, 327-341.

Pérez-Harguindeguy, N., Díaz, S., Cornelissen, J.H.C., Vendramini, F., Cabido, M., Castellanos, A., 2000. Chemistry and toughness predict leaf litter decomposition rates over a wide spectrum of functional types and taxa in central Argentina. Plant and Soil 218, 21-30.

Purohit, J., Chattopadhyay, A., Biswas, M.K., Singh, N.K., 2018. Mycoremediation of agricultural soil: bioprospection for sustainable development, en: Prasad, R. (Ed.), Mycoremediation and Environmental Sustainability. Springer International Publishing AG, Cham, Switzerland, pp. 39-60.

Qi, Y., Zhao, N., Liu, J., Huang, J. 2016. Biochemical responses of ten ectomycorrhizal fungal isolates to manganese. Water, Air and Soil Pollution, 227, 477-487.

Rosling, A., Lindahl, B.D., Finlay, R.D., 2004. Carbon allocation to ectomycorrhizal roots and mycelium colonising different mineral substrates. New Phytologist 162, 795-802.

Saitta, A., Anslan, S., Bahram, M., Brocca, L., Tedersoo, L., 2018a. Tree species identity and diversity drive fungal richness and community composition along an elevational gradient in a Mediterranean ecosystem. Mycorrhiza 28, 39-47.

Strickland, M.S., Osburn, E., Lauber, C., Fierer, N., Bradford, M.A., 2009. Litter quality is in the eye of the beholder: Initial decomposition rates as a function of inoculum characteristics. Functional Ecology 23, 627-636.

Tedersoo, L., Bahram, M., 2019. Mycorrhizal types differ in ecophysiology and alter plant nutrition and soil processes. Biological Reviews 1868, 1857-1880.

Tedersoo, L., Bahram, M., Cajthaml, T., Põlme, S., Hiiesalu, I., Anslan, S., Harend, H., Buegger, F., Pritsch, K., Koricheva, J., Abarenkov, K., 2016. Tree diversity and species identity effects on soil fungi, protists and animals are context dependent. ISME Journal 10, 346-362.

Tedersoo, L., Bahram, M., Põlme, S., Kõljalg, U., Yorou, N.S., Wijesundera, R., Ruiz, L.V., Vasco-Palacios, A.M., Thu, P.Q., Suija, A., Smith, M.E., Sharp, C., Saluveer, E., Saitta, A., Rosas, M., Riit, T., Ratkowsky, D., Pritsch, K., Põldmaa, K., Piepenbring, M., Phosri, C., Peterson, M., Parts, K., Pärtel, K., Otsing, E., Nouhra, E., Njouonkou, A.L., Nilsson, R.H., Morgado, L.N., Mayor, J., May, T.W., Majukim, L., Lodge, D.J., Lee, S.S., Larsson, K.-H., Kohout, P., Hosaka, K., Hiiesalu, I., Henkel, T.W., Harend, H., Guo, L., Greslebin, A., Gretlet, G., Geml, J., Gates, G., Dunstan, W., Dunk, C., Drenkhan, R., Dearnaley, J., Kesel, A. De, Dang, T., Chen, X., Buegger, F., Brearley, F.Q., Bonito, G., Anslan, S., Abell, S., Abarenkov, K., 2014. Global diversity and geography of soil fungi. Science 346, 1256688.

Urbanová, M., Šnajdr, J., Baldrian, P., 2015. Composition of fungal and bacterial communities in forest litter and soil is largely determined by dominant trees. Soil Biology and Biochemistry 84, 53-64.

van der Heijden, M.G.A., Klironomos, J.N., Ursic, M., Moutoglis, P., Streitwolf-Engel, R., Boller, T., Wiemken, A., Sanders, I.R., 1998. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature 396, 69-72.

van der Heijden, M.G.A., Streitwolf-Engel, R., Riedl, R., Siegrist, S., Neudecker, A., Ineichen, K., Boller, T., Wiemken, A., Sanders, I.R., 2006. The mycorrhizal contribution to plant productivity, plant nutrition and soil structure in experimental grassland. New Phytologist 172, 739-752.

Wang, Y.P., Shi, J.Y., Wang, H., Lin, Q., Chen, X.C., Chen, Y.X., 2007. The influence of soil heavy metals pollution on soil microbial biomass, enzyme activity, and community composition near a copper smelter. Ecotoxicology and Environmental Safety 67, 75-81.

Weemstra, M., Mommer, L., Visser, E.J.W., van Ruijven, J., Kuyper, T.W., Mohren, G.M.J., Sterck, F.J., 2016. Towards a multidimensional root trait framework: a tree root review. The New phytologist 211, 1159-1169.

WHO, 2017. Evaluations of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) [WWW Document]. 84th JECFA.

Wilkinson, S.C., Anderson, J.M., Scardelis, S.P., Tisiafouli, M., Taylor, A., Wolters, V., 2002. PLFA profiles of microbial communities in decomposing conifer litters subject to moisture stress. Soil Biology and Biochemistry 34, 189-200.

Wurzburger, N., Hendrick, R.L., 2007. Rhododendron thickets alter N cycling and soil extracellular enzyme activities in southern Appalachian hardwood forests. Pedobiologia 50, 563-576.

Zeglin, L.H., Bottomley, P.J., Jumpponen, A., Rice, C.W., Arango, M., McGowan, A., Mfombep, P., Myrold, D.D., 2013. Altered precipitation regime affects the function and composition of soil microbial communities on multiple time scales. Ecology 94, 2334-2345.

10. CONCLUSIONES

1. Tras 18 años del vertido minero de Aznalcóllar y de la implementación de una estrategia de fitorremediación, la cobertura vegetal establecida en el Corredor Verde del Guadiamar ha producido cambios en la química y en la actividad biológica del suelo.
2. La forestación con las especies leñosas estudiadas (acebuche, álamo blanco y pino piñonero) aumentó el contenido en materia orgánica y la relación C:N, en comparación con las praderas adyacentes. El pino piñonero en sustratos ácidos produjo una acidificación en el suelo, mientras que en suelos neutros los carbonatos tamponaron este efecto.
3. El C y N microbiano mostraron valores más altos en los suelos bajo las especies leñosas, comparado con las praderas adyacentes, especialmente en el sitio Sur. La actividad enzimática fue más influenciada por las propiedades abióticas del suelo (pH, C, N y P) en primavera que en otoño.
4. El álamo blanco fue la especie con una mayor diversidad catabólica del suelo, posiblemente por sus efectos en la materia orgánica y la humedad del suelo, promoviendo el mayor desarrollo de las comunidades microbianas.
5. La cobertura vegetal produjo efectos específicos en el suelo y fue determinante en la composición y estructura de las comunidades fúngicas del suelo. El pH y el contenido en carbono y nutrientes en el suelo fueron determinantes en la diversidad, riqueza específica y equitabilidad de las comunidades fúngicas. Mientras que el pH, la textura y la contaminación fueron determinantes en su composición taxonómica y funcional.
6. En suelos contaminados y no-remediados el establecimiento de la vegetación y de las comunidades fúngicas estuvieron limitados por la elevada contaminación, el bajo pH y la escasez de nutrientes. La diversidad y riqueza específica de hongos fueron mayores en los suelos remediados, debido posiblemente al efecto de la vegetación, aumentando el pH, nitrógeno, carbono orgánico y la relación C:N.
7. En cuanto al efecto de las diferentes especies de árboles, el álamo blanco y el pino piñonero promovieron una mayor diversidad y funcionalidad en las comunidades de hongos del suelo. También compartieron unas comunidades más similares, al ser ambas especies hospedadoras de hongos ectomicorrícicos. En cambio, el

acebuche presentó una menor riqueza de especies y sus comunidades fueron más similares a las encontradas en suelos de pradera, dominadas por hongos saprótrofos.

8. Las propiedades generales del suelo y el nivel de contaminación no explicaron una alta variabilidad de la diversidad taxonómica de especies ectomicorrícicas en la rizosfera de la encina. Sin embargo, la diversidad funcional de las comunidades de hongos ectomicorrícicos, sí estuvo ampliamente explicada por estos factores ambientales. En particular, la concentración de elementos traza en el suelo tuvo un efecto supresor en las estructuras de exploración (hifas emanentes y rizomorfos) y produjo un filtrado ambiental, aumentando la convergencia funcional de los hongos
9. Los rasgos morfológicos y químicos de las raíces y hojas de encina estuvieron determinados en mayor medida por las comunidades de hongos ectomicorrícos y sus rasgos morfológicos (hifas emanentes, rizomorfos y melanina), que por las propiedades abióticas del suelo.
10. Los rasgos de los hongos ectomicorrícicos covariaron con el "espectro económico de la raíz". La formación de rizomorfos y el contenido en melanina fueron rasgos de los hongos relacionados con el gradiente de adquisición-conservación de la raíz. La transferencia de elementos traza del suelo a las hojas fue reducida, gracias al papel protector de los hongos ectomicorrícicos, contribuyendo al potencial fitoestabilizador de la encina.
11. Las setas *Volvopluteus gloiocephalus* y *Laccaria laccata* presentaron diferentes valores de isótopos de C y N, indicando sus tipos de nutrición contrastados: saprótrofo y ectomicorrícico, respectivamente. Ambas especies de setas presentaron una elevada acumulación de Cd y Cu. El consumo de estas setas podría suponer un riesgo de toxicidad por alta concentración de Cd.
12. En suelos contaminados por elementos traza es necesario realizar una continua monitorización y en el caso de establecerse una estrategia de fitorremediación es importante la adecuada selección de las especies forestadas para la recuperación de los servicios ecosistémicos.